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PROTEIN ADP-RIBOSYLATION
IN
TRITICUM AESTIVUS L.

submitted
by
Nazar A.N. Abed

for the degree of Ph.D.
of the
University of Bath
1983

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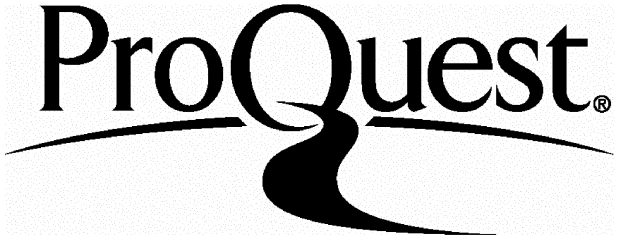

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Abstract

Poly(ADP-ribose) is an unusual polymer formed exclusively in the nuclei of eukaryotic cells. It was discovered in the early sixties in the laboratory of Mandel. The work described in this thesis is aimed at providing an insight into the role of poly(ADP-ribose) in wheat embryos germination (Triticum Aestivus L.). It was shown that nuclei isolated from wheat seeds can incorporate [^{3}H]NAD into acid-insoluble material which was subsequently shown to be poly(ADP-ribose) (Whitby & Whish, 1977). Having established the presence of the polymerising enzyme, ADP-ribose transferase, a method was developed for the isolation of ADP-ribosylated proteins in vivo.

A frozen isolated nuclei system was established and it was shown that poly(ADP-ribose) glycohydrolase activity was inactivated by freezing at -20°C.

Inhibitor studies have also been employed both in vitro and in vivo in an attempt to relate ADP-ribose transferase activity to the rate, and/or the amount, of germination.

The aim of these studies was to find a biological role for protein ADP-ribosylation in the wheat embryo. A comparison could then be made with animal systems so that insight might be gained regarding the function of protein ADP-ribosylation.

D E D I C A T I O N

T o

M y F a m i l y

a n d M y C o u n t r y

ABBREVIATIONS

3-AB	3-aminobenzamide
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribose
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BHK	Baby hamster kidney
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CLL	Chronic lymphocytic leukemia
CNBT	Cyanoborohydride
dADPR	Deoxyadenosine diphosphate ribose
dAMP	Deoxyadenosine monophosphate
3'dATP	3'-deoxyadenosine triphosphate
DMS	Dimethyl sulphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
$dNAD^+$	Deoxynicotinamide adenine dinucleotide
DNase	Deoxyribonuclease
DTNB	Dithionitrobenzoic acid
EAT	Ehrlich ascites tumour
EDTA	Ethylene diamine tetra-acetic acid
EtoH	Ethanol
HMBA	Hexamethylenebisacetamide
MNU	N-methyl-N-nitrosourea
NaAc	Sodium acetate
Na_3CNBH_3	Sodium cyanoborohydride

NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NMN	Nicotinamide mononucleotide
PCA	Perchloric acid
PEI	Polyethyleneimine
PHA	Phytohaemagglutinin
PPO	2,5-diphenyloxazole
PR-AMP	2'(5"-phosphoribosyl)-5'AMP
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
SM	Streptomycin sulphate
SVPDE	Snake venom phosphodiesterase
TCA	Trichloroacetic acid
TEA	Triethanolamine
TEMED	N',N',N',N'-tetramethylethylenediamine
T.L.C.	Thin-layer chromatography
Tris	2-amino-2-hydroxymethyl propane 1,3-diol
UV	Ultra violet

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CHAPTER 1

Introduction

1.1. Discovery and Occurrence of Poly(ADP-ribose)

As early as 1950, Kornberg (1950) purified an enzyme from yeast and liver which catalysed the reversible synthesis of NAD and pyrophosphate (PP) according to the equation:

nicotinamide mononucleotide(NMN) + ATP $\rightleftharpoons$ NAD + PP

A study of the purification and properties of the yeast and liver enzymes and the kinetics of the reaction showed that this enzyme was NAD pyrophosphorylase (Kornberg, 1950). It was reported by Kornberg (1950) that the known enzymatic reactions of ATP involved either a hydrolytic cleavage of one of the pyrophosphate linkages or a transfer of the terminal phosphate group to an acceptor molecule such as adenylic acid, sugar, or protein. Hogeboom et al. (1952) showed that the synthesis of cellular NAD in liver cells is largely confined to the nucleus. They considered that the synthesis of NAD was a function of extraneous cell structures contaminating the nuclear fraction. Zatman et al. (1952) quoted the work of Handler & Klein (1942) which demonstrated an enzyme system in animal tissues which was able to split NAD (See Figure 1.1). The latter molecule contains two energy rich bonds which are involved in group transfer reactions. These two bonds are:

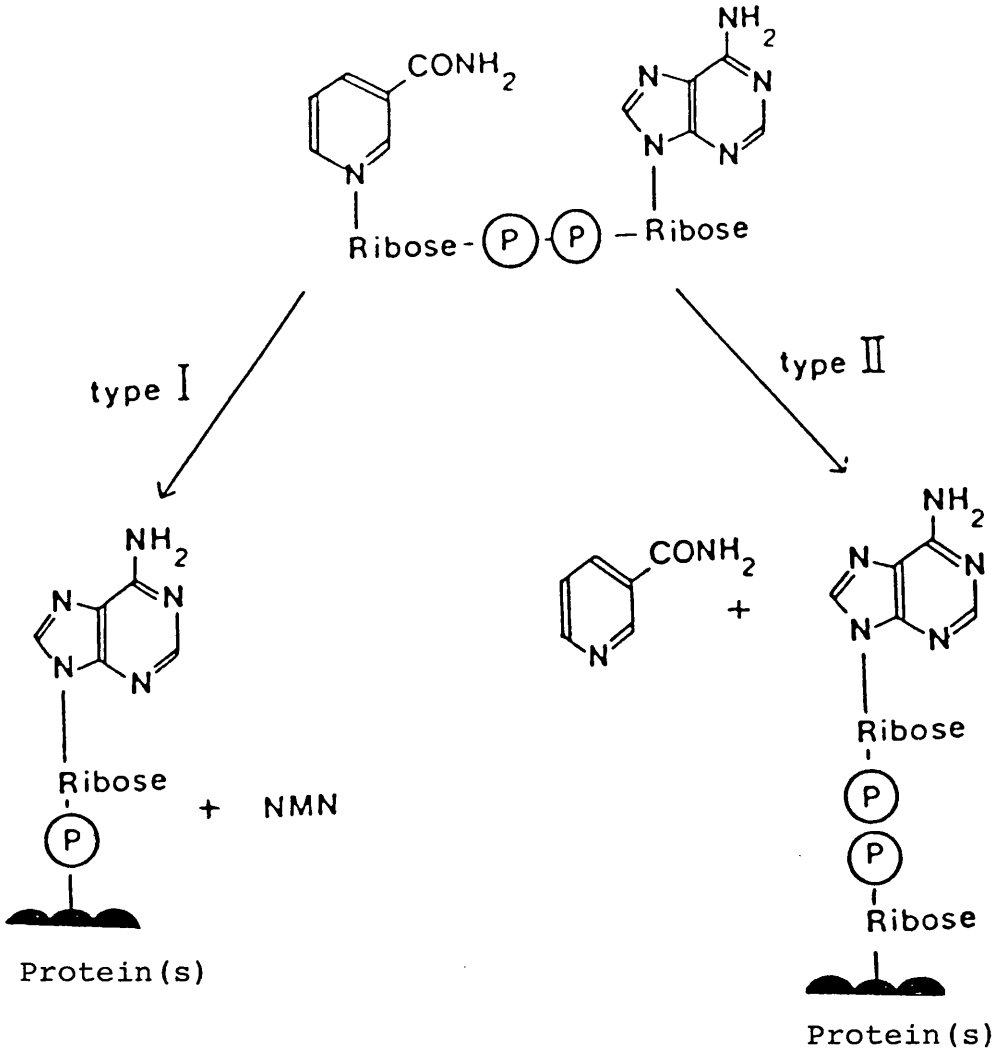


Figure (1.1) Transfer reactions of NAD

a) pyrophosphate bond, which may be cleaved by phosphodiesterase-like enzymes;
b) the nicotinamide riboside bond (glycosidic linkage at the quaternary nitrogen atom of the pyridine ring of the nicotinamide moiety (Zatman et al., 1953). Revel & Mandel in 1962 demonstrated the existence of an enzymic system which catalysed the incorporation of [^{14}C] ATP into an acid-insoluble product. This incorporation was stimulated 1,000-fold on addition of NMN (Chambon et al., 1963). NAD^+ was found to be a better precursor than NMN plus ATP (Chambon et al., 1966; Nishizuka et al., 1967; Sugimura et al., 1967). A digestion of the acid-insoluble material was carried out by Chambon (Chambon et al., 1966) using snake venom phosphodiesterase. This analysis showed that the acid-insoluble material was in fact a polymer of adenosine diphosphate ribose (ADPR).

The formation of poly(ADPR) was catalysed by a transglycosylation enzyme involving the ADPR moiety of NAD^+. During the reaction, the nicotinamide moiety of NAD^+ was found to be released. Poly(ADP-ribose) was shown to be polynucleotide in that it contained adenine, ribose and phosphate (Chambon et al., 1966). However, it differed from other nucleic acids in that it contained ribose-ribose bonds as well as pyrophosphate bonds (see following sections).

The natural occurrence of poly(ADP-ribose) has been studied extensively by many investigators (Sugimura et al.,

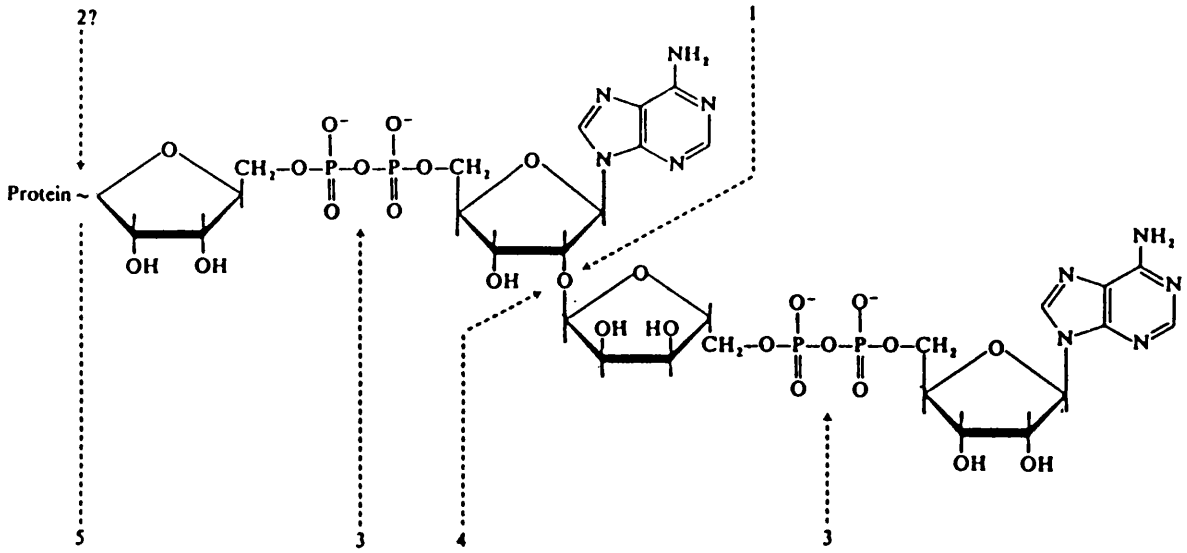


Figure (1.2) The structure and enzymology of poly(ADP-ribose) (chain length 2),

1: ADP-ribose transferase

2: Initiating enzyme

3: Phosphodiesterase

4: Poly(ADP-ribose) glycohydrolase

5: ADP-ribose-protein hydrolase

1973; Hilz & Stone, 1976; Hayaishi & Ueda, 1977). There have been many preliminary reports on the qualitative demonstration of poly(ADP-ribose) *in vivo* but conclusive proof of its natural occurrence was the important problem in earlier studies of its biological function (see Section 5). Poly(ADP-ribose) has been reported to be present in many animal tissues e.g. rat (Nishizuka *et al.*, 1967; Burzio & Koide, 1972), mouse L cells (Hilz & Kittler, 1971; Shall *et al.*, 1972; Lehmann & Shall, 1972), human tissues (Burzio *et al.*, 1971; Preiss *et al.*, 1971), cattle (Ito *et al.*, 1979; Okayama *et al.*, 1977), pig (Lehmann *et al.*, 1974; Janakidevi & Koh, 1974), pigeon erythrocytes (Nishizuka *et al.*, 1967), fish (Sugimura, 1973), birds (Chambon *et al.*, 1966; Muller *et al.*, 1974) and the slime mould Physarum polycephalum (Brightwell & Shall, 1971). Poly(ADP-ribose) has also been reported to be present in plants. Payne & Bal (1976) reported a fixation of [^{3}H] NAD by the nuclei of onion cells, using autoradiographic methods. Lin (1976) reported poly(ADP-ribose) in rice, Whitby & Whish (1977) reported the presence of poly(ADP-ribose) in wheat and finally Laroche & Poirier reported the presence of poly(ADP-ribose) in rye (Larochi & Poirier, 1980).

1.2. Structure of Poly (ADP-ribose)

The structure of poly(ADP-ribose) (see Figure 1.2).

has been investigated by both physical and chemical techniques, notably NMR spectral analysis and specific enzyme digestion. Several workers, Doly & Petek (1966), Chambon et al. (1966), Nishizuka et al. (1967) and Reeder et al. (1967) have reported in detail on the structure of poly(ADP-ribose). They indicated that only one of the two riboses in the ADP-ribose moiety had free adjacent hydroxyl groups at position 2' and 3'. Phosphatase treatment yielded ribosyl-adenosine, and methylation followed by acid hydrolysis demonstrated a 1'-2" glycoside linkage.

Miwa (Miwa et al., 1977) analysed the polymer by NMR and found that it consisted of repeating units of (1"-2')-α-D-ribofuranosyl-adenosine 5',5"-bis (phosphate).

Studies on the chain length of poly(ADP-ribose) formed in isolated nuclei show that the product may have as many as 65 ADP-ribose residues. The chain length of poly(ADP-ribose) was determined in isolated nuclei which had been incubated with [^{3}H]NAD. The product was digested by snake venom phosphodiesterase, and paper chromatography used for resolution of 5'-AMP and PR-AMP. The radioactivity associated with each compound was analysed. The ratio of the total radioactivity (AMP and PR-AMP) to the radioactivity in AMP gave the average chain length (Nishizuka & Ueda, 1969).

The first attempt at resolving polymers of different sizes was that of Sugimura et al (1971). Isolated nuclei

labelled with [^{14}C]NAD^{+} were digested with pronase (to free polymer from protein) and the remaining DNA, RNA and poly(ADP-ribose) subjected to hydroxyapatite chromatography, elution was effected with increasing concentration of phosphate buffer. A direct relationship between chain length and phosphate concentration as well as a good separation of poly(ADP-ribose) from DNA and RNA was observed. The same group later analysed the observed peaks by gel electrophoresis and found each peak was comprised of two subfractions differing in both chain length and terminal structure (Tanaka et al., 1977). They suggested that the latter may be due to partial degradation by hydrolytic enzymes. Tanaka et al. (1978) modified their earlier method of fractionation using a combination of hydroxyapatite chromatography and gel electrophoresis, to determine the presence of at least 65 discrete species of polymer. Analysis of the chain length of each band by the conventional method of Nishizuka et al. (1969) showed chain lengths of up to (ADP-ribose)$_{30}$. They suggested that this may be due to the presence of a branched structure with two or more AMP termini. Miwa et al. (1979) extended this work by analysing the hydrolysis products of a snake venom phosphodiesterase digest on the high molecular weight polymer fraction obtained from hydroxyapatite chromatography. By using a combination of thin-layer chromatographic and mass spectrometric techniques,

the existance of a new compound 2'[1"-ribosyl-2"(or 3")-1"'ribosyl] adenosine-5',5",5"'-tris (phosphate) which constituted 2% of the reaction products, was demonstrated. This finding provided evidence in support of a branched structure whereby additonal (ADP-ribose) molecules are added, via a 1"'-2" or 1"'-3" glycosidic linkage, to the ribose on which nicotinamide was originally attached. Farzaneh & Pearson (1978) used the hydroxyapatite column chromatography method to determine the chain length of poly(ADP-ribose) during development of Xenopus laevis. They found that this method gave larger estimates than the phosphodiesterase method of Nishizuka et al. (1969). They proposed that [^{3}H]NAD was incorporated in vitro onto pre-existing chains synthesised in vivo. The same group reported a similar result after analysing the reaction product from BHK cells (Furneaux & Pearson, 1980). Analysis of poly(ADP-ribose), using a variety of different tissues and species, revealed that the chain length of the polymer varied substantially.

Adamietz & Hilz (1976) showed that on caesium sulphate density gradient centrifugation, poly(ADP-ribose) from a variety of tissues exhibited a range of different sizes. Adamietz et al. (1978) demonstrated quantitative differences in the chain length pattern of nuclei from different tissues. They used the high resolution of polyacrylamide gel electrophoresis and the susceptibility

of the protein-poly(ADP-ribose) linkage to alkali (Nishizuka et al., 1969) to analyse individual polymer chains obtained from [^{3}H]NAD^+-labelled nuclei. Chain lengths of up to 33 units were resolved, the size distribution being visualised by fluorography and quantified by counting the radioactive content of each gel slice.

1.3. Synthesis of Poly(ADP-ribose)

The synthesis of poly(ADP-ribose) may involve one or more enzymes (see Figure 1.2). The general reaction of the synthesis is:

$$n\ NAD^+ \longrightarrow (ADP\text{-}ribose)n + nNM + nH^+$$

The enzyme which catalyses the transfer of ADP-ribose moiety from NAD^+ to form poly(ADP-ribose) is ADP-ribose transferase although it is often called, less correctly, poly(ADP-ribose)polymerase or poly(ADP-ribose)synthetase. The substrate for this enzyme was shown to be NAD^+ (Chambon et al., 1966) (see Section 1.1) and since then, the ADP-ribose transferase has usually been assayed using the incorporation of radioactive substrate (NAD) into acid-insoluble material. ADP-ribose transferase has been characterised from a wide range of eukaryotic organisms (See section 1.1) and the majority of characterisations have been carried out using isolated nuclei. ADP-ribose

transferase has a specific requirement for NAD. $NADH_2$, NADP, $NADPH_2$ and α-NAD do not act as substrates (Chambon et al., 1966). The specificity for β-NAD was high. Suhadolnik et al. (1977) examined the effect of various NAD analogues with altered adenine or ribose moieties. Nicotinamide tubercidin dinucleotide, 2'(AMP)deoxy-NAD and 3'(AMP) deoxy NAD were all incorporated, but the average chain length was lower than that using NAD.

1.4. Purification of ADP-ribose Transferase

Extensive studies have been carried out in an effort to purify the transferase itself. The first attempts at purification basically involved isolating chromatin and then separating the transferase from DNA and other macromolecules by a combination of chromatographic techniques and ammonium sulphate precipitation (Yoshihara, 1972; Okayama et al., 1977). Okayama et al. (1977) found that the enzyme was very unstable after its separation from DNA. The first attempt to use affinity chromatography was reported by Khan & Shall (1976). A purification of 85-fold was achieved by coupling nicotinamide (an inhibitor of the transferase) or Dextran blue (which has a chromophore structure similar to NAD^+) to Sepharose. In this case more than 100% yield was obtained which suggested the removal of an endogenous inhibitor or degradative

enzymes during the purification step.

A variety of groups have studied the effect of DNA and histones on the activity of preparations of ADP-ribose transferase. DNA is known to stimulate ADP-ribosylation when added to enzyme extracts. Kristensen & Hotlund, (1976) studied the affinity of the transferase for DNA, in an attempt to purify the enzyme. They prepared a high specific activity transferase by employing a two-step procedure which consisted of phosphate extraction and DNA-agarose chromatography (Yoshihara et al., 1978). An additional isoelectric focussing step was later included (Kristensen & Hotlund, 1978). Ito et al. (1979) also used a DNA affinity step and they were able to purify the transferase to apparent homogeneity. Tsopanakis et al. (1978) observed that their final enzyme preparation was extremely labile. To minimize inactivation during purification, they employed an organic solvent, 50% ethylene glygol, at -10°C to achieve higher specific activity.

The molecular weight of the transferase has been determined using sedimentation equilibrium, gel electrophoresis and varies from 63,000 in pig thymus (Tsopanakis et al., 1978) to 130,000 in calf thymus (Yoshihara et al., 1978). It is not known whether these different values in molecular weight reflect real differences in the structure of the enzymes or arise during the different purification steps involved. Sedimentation data suggests that the

enzyme is a globular protein with slight asymmetry (Ito et al., 1979). The enzyme has a pI of 9.8 and amino acid analysis showed that the transferase contained a high proportion of residues that are either acidic or basic and that the N-terminal appeared to be blocked. This is in agreement with the data of Tsopanakis et al. (1978) for the pig thymus enzyme. This latter feature has been reported for other chromatin proteins, including the high mobility group proteins (Goodwin & Johns, 1977) and a non-histone protein from hen oviduct (Teng et al., 1978). Hotlund & Kristensen (1980) obtained similar results to those for calf thymus when they characterised the enzyme from Ehrlich ascites tumor (E.A.T.) cells, except for differences in the sedimentation coefficient which gave an apparent molecular weight of over 500,000. They suggested the active enzyme may be an oligomer.

ADP-ribose transferase was found to be located exclusively in the nucleus of the cell (Stocken, 1978). The combination of cellular and nuclear fractionation showed that at least 90% of the activity was associated with chromatin (Nishizuka et al., 1969) and this activity was strongly bound to DNA (Ueda et al., 1968). Hilz & Kittler (1968) also demonstrated by autoradiography that ADP-ribose transferase was present in nuclei with little activity in the nucleoli. Furneaux & Pearson (1980) have confirmed these original findings. Mitochondria have been

reported to possess ADP-ribose transferase activity (Kun et al., 1975). This differs from the nuclear enzyme in that mono ADP-ribose is transferred to a sinlge protein and this activity is unaffected by thymidine (an inhibitor of nuclear enzyme - see below). Koide also reported an enzyme similar to the enzyme in the mitochondria of Xenopus Laevis following germinal vesicle breakdown (Burzio et al., 1979).

The activity of the transferase in isolated nuclei has shown to be stimulated by, but not dependent on, Mg^{2+} and a thiol reagent. Stone & Shall (1973) reported that the stimulation of the enzyme by Mg^{2+} in LS cells was dependent on NAD^{+} concentration. The stimulation of the enzyme by thiol reagents suggests that the enzyme contains SH groups in reduced form for maximal activity. The enzyme does not obey normal Michaelis-Menten kinetics (Furneaux & Pearson, 1980; Whitby, 1980). The affinity of the enzyme for NAD^{+} seems to differ between tissues, and may vary at different stages of the cell cycle (Brightwell et al., 1975). The enzyme from both BHK (Furneaux & Pearson, 1980) and wheat embryos (Whitby, 1980)is activated at high substrate concentrations. In contrast, the enzyme from rat pancreas is apparently inhibited by high concentrations of NAD^{+} (Poirier et al., 1978). Km values obtained have varied from 23μM in pig thymus (Tsopanakis et al., 1978) to 1.5mM in LS cells

(Stone & Shall, 1973). Two different Km values were obtained for the enzyme from HeLa cells in log and stationary phase (Kidwell et al., 1974), and two different Km values were obtained for the enzyme involved in the formation of hydroxylamine-sensitive and resistant ADP-ribosylation. These values have been estimated under a variety of different conditions and purification states. This might well account for the difference in values obtained.

1.5. Inhibitors of ADP-ribose Transferase

Synthesis of poly(ADP-ribose) in isolated nuclei is inhibited by various groups of compounds such as substrate analogues like α-NAD and $NADH_2$ which have been reported to be good inhibitors (Hilz & Stone, 1976). Other inhibitors include nicotinamide and 5-methylnicotinamide which are competitive end-product inhibitors (Clark et al., 1971; Preiss & Hilz, 1971). The enzyme is also inhibited by thymidine and some of its analogues (Preiss et al., 1971). Levi et al. (1978) have shown that methylated xanthines and cytokinins are also good inhibitors. Purnell, (1981) have shown that the most important and potent inhibitors are the 3-substituted benzamides. 3-Aminobenzoic acid and various acetophenones were good inhibitors of ADP-ribose

transferase. Furneaux & Pearson (1980) showed that 2-amino-1,3,4-thiadiazole also inhibited the enzyme. Yamamoto & Okamoto (1980) have shown that picolinamide (an isomer of nicotinamide) was an inhibitor of the transferase. The latter inhibition was almost identical to that of nicotinamide but, unlike nicotinamide, picolinamide is not part of the NAD molecule. However, most of the above compounds, as well as other inhibitors of ADP-ribose transferase are known to affect other cellular processes. Nicotinamide affects the synthesis of NAD^+ and may therefore also affect the synthesis of poly(ADP-ribose). Berger & Sikorski (1980) demonstrated that nicotinamide stimulated repair of DNA damage, possibly through poly(ADP-ribose), in human lymphocytes. Finally, polar compounds including $(NH_4)_2SO_4$ (Whitby, 1980) have been found to inhibit the enzyme while non-polar compounds stimulated it (Kristensen & Hotlund, 1978). Polyamines have been shown to stimulate ADP-ribosylation in isolated nuclei from various tissues (Muller & Zahn, 1976 ; Tanigawa et al., 1977; Perella & Lea, 1978, 1979). A differential effect was reported with the presence or absence of Mg^{2+}. The effect of polyamines cannot be explained by inhibition of degradative enzymes, as it was reported by Whitby & Whish (1979) that polyamines did not inhibit poly(ADP-ribose) glycohydrolase, an enzyme which hydrolyses the poly(ADP-ribose) in isolated nuclei from wheat seed (Section 2.6). Polyamines appear to cause changes in ADP-ribosylation by altering the

accessibility of acceptor proteins rather than by stimulating poly(ADP-ribose)transferase.

Halldorsson et al. (1978) and Berger et al. (1978) developed nucleotide-permeable cell systems with which to perform ADP-ribose transferase. They reported that this system reflected enzyme activity in vivo more accurately than isolated nuclei. A variety of groups have studied the effect of histones on the activity of preparations of ADP-ribose transferase. Histones were reported to stimulate the activity of purified enzyme 2- to 3-fold in the presence of DNA (Okayama et al., 1977). The stimulation was found to increase the chain length of poly(ADP-ribose) but not the number of chains synthesised (Okayama et al., 1977; Ito et al., 1979). Okayama et al. (1977) found that histones stimulated ADP-ribose transferase activity but were unsuccessful in detecting the ADP-ribosylation of histones when the reaction was analysed by gel electrophoresis. They later showed that the purified enzyme preparation could incorporate ADP-ribose onto a mono(ADP-ribose)-H1 conjugate (Ueda & Hayaishi, 1979). This suggested either that there might be two separate enzymes (one for initiation and the other for elongation) or, that this activity was lost during the purification procedure. The same group (Kawaichi & Hayaishi, 1980) reported recently that, by using higher H1 and DNA levels than previously, the enzyme could both initiate and elongate

oligo(ADP-ribose)-histone H1 conjugates. Thus it seemed that the purified transferase was capable of catalysing two different reactions, the formation of a glycosidic and an ester bond and, as a result, catalysed both chain elongation and chain initiation. Yoshihara et al. (1977) reported that the purified enzyme was capable of initiating self-ADP-ribosylation in the absence of any exogenous acceptor protein. Analysis by gel filtration and SDS poly-acrylamide gel electrophoresis showed that the enzyme and radioactivity were coincident on a single peak. They found that the bound radioactivity was oligo(ADP-ribose) with an average chain length of 2.6. It was also shown that the linkage of the enzyme-bound early product was covalent since it was stable in SDS and urea. By lengthening the incubation time, Yoshihara et al., (1977) found that the polymer and the enzyme became separate on SDS polyacrylamide electrophoresis. This may have indicated that on reaching a certain length, the polymer was released from the enzyme which then allowed the initiation of a new chain. The chain may be released and transferred to the acceptor protein after varying lengths of time depending on the structure of that protein.

Mandel's group (1979,1980) compared the purified enzyme preparation before the removal of active DNA with a reconstituted preparation. The DNA-independent enzyme before application to hydroxyapatite chromatography was able to ADP-ribosylate all the histones. It was also able to

modify itself as well as purified cholera toxin, E.coli polymerase and calf thymus RNA polymerase B. However calf thymus RNA polymerase A or C were not substrates (Niedergang et al., 1979; Okazaki et al., 1980). In contrast, after the DNA dependent enzyme had been chromatographed on hydroxylapatite, the only histone it was able to ADP-ribosylate was H1 (Okazaki et al., 1980). Tanaka et al. (1979) found that a purified enzyme could only ADP-ribosylate histone H1 if Mg^{2+} was omitted from the reaction mixture.

A nucleotide permeable cell system was developed for measuring the enzyme because the activity obtained from isolated nuclei was an overestimation. This was found to be due to the activating effect of the damaged DNA on the enzyme (Halldorsson & Shall, 1978). Shall's group showed that the activity in nucleotide permeable cells was much lower than in an equivalent number of isolated nuclei. If these cells were pre-incubated in absence of NAD, the enzyme activity increased until it approached those values found in isolated nuclei. It is noteworthy to mention that plant cells cannot be permeabilized due to their thick cell walls; no permeabilisation technique applicable to plant cells has been described in the literature.

1.6. Degradation of poly(ADP-ribose)

There are two enzymes implicated in the degradation

of poly(ADP-ribose). The first is a phosphodiesterase which hydrolyzes the pyrophosphate bonds (see Figure 1.2) releasing 2'-(5"-phosphoribosyl)-5'-AMP(PRAMP) (Futai et al., 1967). The second is an exoglycosidase which splits the 1'-2" ribose-ribose and is called poly(ADP-ribose) glycohydrolase (Miwa & Sugimura, 1971; Ueda et al., 1972 ; Tanaka et al., 1976).

Venom phosphodiesterase hydrolyzes poly(ADP-ribose) endonucleolytically (Matsubara & Sugimura, 1970). The final products of digestion are phosphoribosyl-AMP from the internal residues of poly(ADP-ribose) and 5'-AMP from the terminus. Snake venom phosphodiesterase has proved to be a very useful tool in the identification and characterisation of poly(ADP-ribose). This is because of the unique compound, phosphoribosyl-AMP, which is produced on SVPDE digestion of the polymer. Rat liver phosphodiesterase hydrolyzes poly(ADP-ribose) exonucleolytically (Futai et al., 1968) from the AMP terminus (Matsubara et al., 1970b) producing first AMP then phosphoribosyl-AMP. This enzyme was also found to be capable of hydrolysing NAD^+ and ADPR.

The polymer is cleaved at the ribose-ribose bond by poly(ADP-ribose) glycohydrolase which was first found in calf thymus (Miwa & Sugimura, 1971, 1974). Enzymes with similar specificity were found in normal organs and tumors of rats (Ueda & Hayaishi, 1972; Miwa & Sugimura, 1975) in

wheat embryo (Whitby & Whish, 1978) and in Physarum polycephalum (Tanaka & Shall, 1976). Poly(ADP-ribose) glycohydrolase plays an important role in hydrolysis of poly(ADP-ribose) (Miwa & Sugimura, 1975). The mode of hydrolysis by the enzymes of calf thymus and Physarum polycephalum was exoglycosidic (Miwa & Sugimura, 1974; Tanaka & Shall, 1976). After hydrolysis of polymer by poly(ADP-ribose) glycohydrolase, it may be re-elongated by ADP-ribose transferase. This means that the glycohydrolase may play a role in regulating the size of poly (ADP-ribose). The glycohydrolase appears not to cleave the linkage between ADP-ribose and protein since little hydrolysis of histone-bound mono(ADP-ribose) was observed (Ueda et al., 1972). This observation suggests that an additional enzyme, a 'hydrolase' is needed to cleave this last residue from protein. Poly(ADP-ribose)glycohydrolase was shown to be inhibited by DNA (Hilz & Stone, 1976). They showed that denatured DNA is an extremely potent inhibitor of the enzyme. Double-stranded DNA has a variably inhibitory effect on the enzyme that may simply reflect the single-stranded content of the DNA preparations used. Stone et al. (1978) showed that the enzyme was bound more tightly to denatured DNA-cellulose than to double-stranded DNA-cellulose. Inhibition of the enzyme by high concentrations of DNA could be overcome by either increasing the ionic strength of the medium or by adding histone

H1. They suggested that both of these treatments may be displacing the enzyme from inhibitory (possibly single-stranded) portions of DNA. Some properties of the glycohydrolase have been confirmed by purification studies which have allowed further characterisation of this enzyme. Poly(ADP-ribose)glycohydrolase was also reported to be sensitive to ionic strength, being completely inhibited by 0.5M $(NH_4)_2SO_4$ (Stone et al., 1973; Miyakawa et al., 1972). It is also inhibited, non-competitively, by cyclic AMP with an apparent Ki of 1.5mM in rat liver (Ueda et al., 1972; Miyakawa et al., 1972; Stone et al., 1973) and by its end-product, ADPR (Miyakawa et al., 1972). The optimum pH of the enzyme varies between 6.0 and 7.5 for Physarum polycephalum and calf thymus, respectively. The molecular weight is about 50,000 and the enzyme also requires SH groups for activity.

1.7. Chemical and Physical Properties of Poly(ADP-ribose)

Many of the general properties of poly(ADP-ribose) are similar to polynucleotides, in that they are soluble in water, neutral and alkaline solutions. Poly(ADP-ribose) is precipitated by 5% (w/v) TCA or 60% (v/v) EtOH (Fujimura & Sugimura, 1971). It also precipitates in the presence of Mg^{2+} (Doly, 1968). The polymer is relatively stable in alkali and little of the acid-insoluble

material becomes acid soluble on treatment with 0.5M NaOH at 37°C for 18 hours. However, boiling in 1M HCl for 7 minutes results in total conversion to acid-soluble material (Hasegawa et al., 1967). Acid treatment splits the adenine-ribose bond, yielding adenine and ribose 5-phosphate (Reeder et al., 1967). Poly(ADP-ribose) is not hydrolysed by DNase I, DNase II, pancreatic RNase, RNase T, micrococal nuclease, spleen phosphodiesterase, spleen NAD nucleosidase or trypsin (Chambon et al., 1966; Hasegawa et al., 1967; Reeder et al., 1967). Poly(ADP-ribose) is readily hydrolysed by the venom phosphodiesterase from Crotalus adamanteus. In fact, the chain length of poly(ADP-ribose) can be determined from the molar ratio of phosphoribosyl-AMP to 5'-AMP plus 1 after complete hydrolysis of the polymer by snake venom phosphodiesterase (See Section 1.2), since 5'-AMP can only be derived from a terminus (see last reference):

The buoyant density using caesium sulphate equilibrium density gradient centrifugation is 1.75 which is slightly lighter than transfer RNA (Hasegawa et al., 1967).

1.8. Biological Function of Poly(ADP-ribose)

Since the original discovery of poly(ADP-ribose) in rat liver nuclei (Chambon & Mandel, 1966; Sugimura et al., 1967) and particularly since the finding that it is associated with chromatin (Nishizuka & Hayaishi, 1968), this polymer has been expected to play some regulatory role in nuclear functions. Additional support for such an expectation was provided by Morton's earlier proposal that the cellular concentration of NAD, which is very low in rapidly growing cells, might be a controlling factor in the initiation of cell division (Morton, 1958, 1961). The precise biological function of poly(ADP-ribose) or poly ADP-ribosylation of nuclear proteins is still unclear. Most of the information available is circumstantial and fragmentary, and in many cases contradictory results have been reported. Nevertheless, the accumulated evidence seems to indicate a close relationship between poly ADP-ribosylation and chromatin activities. The earliest approach to determining the polymer function was to isolate nuclei from cells in various states and attempt to correlate the activity of the enzyme with a particular cell state. Enzyme-activity measurements from isolated nuclei must be treated with caution. The permeabilized-cell system might provide a more realistic model for the study of

enzyme activity than the use of isolated nuclei. Another approach taken to determine the role of ADP-ribosylation was the use of inhibitors of ADP-ribose transferase *in vivo*. The use of nicotinamide, 5-methylnicotinamide, thymidine and methylated xanthines as physiological inhibitors must be viewed with caution because all these inhibitors are known to affect a number of other cellular processes as well as ADP-ribosylation (Purnell *et al.*, 1980). The major problem in discovering the biological function of poly(ADP-ribose) lies in correlating the data obtained by different methods with a definite function; for example, during differentiation of a particular cell, the rate of DNA synthesis will alter, the patterns of DNA transcription will change and the cell may enter a different stage of its cell cycle.

The function of poly(ADP-ribose) and poly ADP-ribosylation will be clearer when we understand the cellular metabolism and localisation of poly ADP-ribose itself. Experiments on these problems are now in progress using for example, sensitive radioimmunoassay systems and also specific fluorescent tags in an effort to determine the function of poly (ADP-ribose) (Sugimura et al., 1980). Although the functions of this polymer are not yet clear, there is evidence that poly(ADP-ribose) synthesis may be involved in the following cellular

processes:-

1. Cell proliferation
2. Cell differentiation
3. Cell cycle
4. DNA replication and synthesis
5. DNA transcription
6. DNA repair and fragmentation

These processes and the possible involvement of protein ADP-ribosylation in them are now described in detail.

1.8.1. Cell Proliferation

Morton (1961) found that the levels of pyridine nucleotide are low in tumours and in tissues which undergo rapid cellular proliferation. Also a significantly higher activity of ADP-ribose transferase has been noted by many investigators in the nuclei of dividing cells compared with resting cells: hepatomas versus normal controls (Hilz et al., 1974); regenerating versus resting liver (Leiber et al., 1973); mitogen-stimulated versus unstimulated lymphocytes (Roberts et al., 1973; Lehmann et al. 1974); leukemic versus normal white blood cells (Burzio et al., 1975); hormone-stimulated versus unstimulated oviducts (Muller et al., 1974); SV40-transformed versus untransformed cells (Sugimura et al., 1976) and in serum-stimulated versus normal fibroblasts (Furneaux & Pearson, 1978). An extractable enzyme activity higher in

proliferating mouse tissues than in static mouse tissues was also demonstrated (Gill, 1972). In some cases e.g. in neonatal versus adult rat liver (Leiber et al., 1973) and AH-130 hepatoma versus adult liver (Yamada et al., 1973) the activities of ADP-ribose transferase were the same. Furthermore, no direct correlation between ADP-ribose transferase activity and DNA content or synthesis could be observed in a number of other cells (Leiber et al., 1973; Hilz et al., 1974; Lehamann et al., 1974). It seems that the only case where ADP-ribose transferase activity consistently increased was on transition of cells or tissues from the non-growing status to the growing status, as on partial hepatectomy (Leiber et al., 1973), phytohaemagglutinin (PHA) (Lemann et al., 1974), serum (Furneaux & Pearson, 1978) or oestrogen (Muller et al., 1974)stimulation. These observations suggested that ADP-ribose transferase is not just a structural component of chromatin. Furthermore, the higher activites of ADP-ribose transferase characteristic of rapidly proliferating cells, compared with non-proliferating cells, suggested that the polymer might be involved in some stages of cell proliferation.

Adamietz et al (1974) tried to correlate endogenous levels of mono and poly(ADP-ribose) in tissues with different proliferation rates. They analysed the ratio of NH_2OH-sensitive oligo(ADP-ribose) to mono(ADP-ribose) residues in proliferating versus non-proliferating tissues.

On quantitation it was found that, although total and NH_2OH-sensitive oligo(ADP-ribose) residues did not differ in the two types of tissue, significantly lower levels of protein-bound NH_2OH-sensitive mono(ADP-ribose) residues were found in proliferating versus non-proliferating tissues. The same result was also found *in vivo* using permeabilised Ehrlich ascites tumour cells (EAT) (Stone & Hilz, 1975). These results were substantiated when ADP-ribose transferase activity was found to be closely related to changes in endogenous mono(ADP-ribose) protein conjugate levels during progression from the proliferating to the non-proliferating state (Bredehorst *et al.*, 1979). It was then observed that there was an increase in both ADP-ribose transferase activity and NH_2OH-resistant mono ADP-ribosylated proteins. Thus there was no obvious direct correlation between ADP-ribose transferase activity and cell proliferation. It may be that the development of methods for the determination of levels and types of ADP-ribosylated protein *in vivo* will elucidate the relationship between the level of transferase activity and cell proliferation.

1.8.2. Cell Differentation

Caplan & Rosenberg (1975) studied the differentiation of mesodermal cells of chick-embryo limb buds into either muscle or cartilage in an attempt to find a

relationship between cell differentiation and observed fluctuations in the cellular levels of NAD^+ and poly (ADP-ribose). Previous work from Caplan's laboratory had shown a correlation between pyridine nucleotide concentrations and cellular differentiation, and they suggested that fluctuations in cellular NAD^+ concentration might play a role in the control of muscle as against cartilage development. They showed that 3-acetylpyridine potentiated cartilage differentiation and this was associated with a stimulation of the rate of poly(ADP-ribose) synthesis. Administration of 3-acetylpyridine had previously been shown to decrease NAD concentrations, and the suggested mechanism was that such a change in NAD^+ levels was 'sensed' and resulted in differential rates of ADP-ribosylation that were correlated with differentiation into either muscle or cartilage (Caplan & Rosenberg, 1975). This proposal was questioned by McLachlan et al. (1976) who showed that the effect of 3-acetylpyridine was mediated via a destruction of peripheral nerves, resulting in a total loss after 24 hours as well as a deleterious effect on all cell types, including cartilage, tendon, mesenchyma and muscle. These results suggest that 3-acetylpyridine affects muscle growth rather than muscle differentiation, as does the observation that 3-acetyl-pyridine still has an effect on muscle tissue at stages when cartilage and muscle cells are physically separated (McLachlan et al., 1976).

A differential effect on poly(ADP-ribose) levels was also found in hormone-induced differentiation in quail oviduct; ADP-ribose transferase activity increased on oestrogen treatment, concomitant with DNA and cell replication and decreased on progesterone treatment and subsequent avidin synthesis (Muller et al., 1974).

Since these observations, several reports have appeared suggesting an involvement of poly(ADP-ribose) in differentiation. Burzio & Koide (1977) showed that during oocyte maturation in Xenopus bevis when mediated by progesterone, a dramatic condensation of the chromosomes occurred just after the breakdown of the oocyte nucleus. It has been shown that during such maturation ADP-ribose transferase activity in isolated nuclei increases 3-fold, this increase occurs before the breakdown of the oocyte nucleus (Burzio & Koide, 1977). Farzaneh & Pearson (1979) also showed a substantial increase in transferase activity during the embryonic development of Xenopus. These results strongly suggest that ADP-ribosylation is directly involved in the early differentiation of Xenopus.

The incorporation of [^{3}H] adenosine into RNA and ADP-ribosylated protein in mouse ova has been studied by Young & Sweeney (1978). They showed that the total incorporation is maximal at three to five hours after ovulation, which is the time of normal fertilisation. The incorporation is low at seven to eight hours at which time

the ability of the ovum to be fertilised was decreased. This suggests a possible role between adenosine metabolism mediated in part through poly ADP-ribosylation, and post-ovulation maturation of the ovum and its fertilizability. The same workers (Young & Sweeney, 1979) also showed that ADP-ribosylation occurred in the mouse one-cell embryo and that this differed in both average chain length and lability of the protein-ADP-ribose bond compared with that in the unfertilised ovum. Such differential ADP-ribosylation in the ovum and very early embryo may indicate that a differentiation-related programme of poly(ADP-ribose) synthesis may be taking place in this system.

In an attempt to correlate differentiation with poly(ADP-ribose), Hilz et al. (1980) also analysed protein-bound ADP-ribose levels. By quantitating mono(ADP-ribose) protein stages of gene expression, they found by radioimmunoassay, that the state of differentiation may determine the endogenous levels of protein-ADP-ribose conjugates.

An indirect immunofluorescene technique was used by Ikai et al (1980) who showed that poly(ADP-ribose) was synthesised in nuclei of lymphocytes and monocytes in normal blood but not in granulocytes or erythrocytes. In addition poly(ADP-ribose) synthesis was detected in myelocytes (precursors of granulocytes) of patients with acute myeloblastic leukemia as well as patients in

blastic crisis of chronic myelocytic leukemia but not in normal blood or bone marrow. These findings suggested that the capacity for synthesising poly(ADP-ribose) may serve as a marker of granulocyte differentiation (Ikai et al., 1980). It has been shown by Yamada et al. (1978) that mouse myeloid leukaemic cells can be induced to differntiate into cells with phagocytic activity , Fc receptors and lysozome activity by the addition of poly(ADP-ribose) to the culture medium and that a certain proportion of these cells differentiate further into granulocytes and macrophages. The addition of dextran sulphate or poly vinyl sulphate was also effective in the induction of phagocytic cells. The physiological significance of the observed stimulation of differentiation by poly(ADP-ribose) must remain questionable, although the radioactivity derived from the polymer enters the cells and becomes located in the nucleus and nuclear membrane, as shown by radioautography methods.

Using F4N friend cells, Rastl & Swetly (1978) showed that, on incubation of erythropoetic differentiation by butyrate, ADP-ribose transferase activity increased three-fold and this affected the ADP-ribosylation of histones (Zlatanova & Swetly, 1980), Morioka et al., 1979 , using cell line 745 showed that poly(ADP-ribose) synthesis supprssion is detectable in the early-exponential-phase growth stage (14-24h) after treatment with hexamethylenebisacetamide (HMBA) or dimethyl sulphoxide

(DMSO). With butyrate, a transient increase in ADP-ribosylation in the early stages of cell growth was seen, but by 48 and 72 hours, ADP-ribosylation was at the same low rate as for hexamethylenebisacetamide and dimethyl sulphoxide treated cells. Nicotinamide also induced differentiation and enhanced dimethyl sulphoxide and hexamethylenebisacetamide-induced differentiation. Friend-cell variants unresponsive to hexamethylenebis-acetamide and dimethyl sulphoxide did not exhibit as low ADP-ribose transferase activity as was found with normal responsive cells treated with inducers. The effect of the inducers was not through a change in cell growth rate or poly(ADP-ribose) degradation. The differences between these two groups may be attributed to either the differences in cell line used-F4N was extremely sensitive to butyrate but relatively insensitive to HMBA (Moroika et al., 1979) or the difference in the method of cell culture. Rastl & Swetly used fresh medium every day whereas Moroika et al. (1979) did not.

The results indicated that there is a clear connection between ADP-ribosylation and the processes involved in differentiation. Whether ADP-ribosylation is involved in the direct control of such processes or is a result of some other aspect of control, and whether the ADP-ribosylation is enhanced or suppressed is still unknown at this time.

detect levels of poly(ADP-ribose) in HeLa cells. They found a six-fold increase in poly(ADP-ribose) as cells traversed from early to late S phase, followed by a rapid decrease and a ten-fold increase at the S-G_2 transition point. The levels obtained correlated with ADP-ribose transferase activity. However more information on the specificity of the antibody was required because the chain length of poly(ADP-ribose) may vary throughout the cell cycle, and long chain polymer may compare much more effectively for antibody binding than oligo(ADP-ribose) or mono(ADP-ribose).

When permeabilised LS1210 cells were treated with cytosine arabinoside (which causes accumulation of cells in G_2 phase), Berger et al. (1978) found that the activity of ADP-ribose transferase increased. When they used Chinese-hamster ovary cells synchronised by mitotic selection, they found that the activity of the enzyme increased during G_1 phase, decreased rapidly as the cells traversed S phase and increased during G_2, M and G_1 phases . When the transferase activity was measured in permeabilised cells after deoxyribonuclease treatment (which indicates total enzyme activity) the level of transferase was constant during the cell cycle except for a small peak during late S-phase which subsequently decreased during the G_2 and M phase (Berger et al., 1976). They suggested that the enzyme was synthesised during S phase and returned to

normal levels after mitosis. DNA damage which occurs during isolation of nuclei may be equivalent to the above DNase treatment in that total or near total ADP-ribose transferase activities are expressed.

Hilz's group (Wielckens et al., 1979) exploited the natural synchrony of the multinuclear plasmodia of the slime mould Physarum polycephalum to study levels of protein-bound mono(ADP-ribose) during the cell cycle. Quantitation, using a sensitive radioimmunoassay, showed that total mono(ADP-ribose) levels decreased from mitosis through S phase and then increased through G_2 phase to M phase. Hydroxylamine-resistant residues increased during S phase, remained constant at the S/G_2 boundary and then decreased at mid G_2. Hydroxylamine-sensitive residues increased at the S/G_2 boundary, then decreased at mid-G_2 followed by a sharp increase and decrease before and after mitosis, respectively. These results may indicate that nuclear ADP-ribosylation reactions serve more than one function. It appears that the permeabilised cell/mitotic selection system of Berger et al. (1978) and the analysis of protein-bound ADP-ribose by Wielckenset al. (1979) may best reflect the changes in ADP-ribosylation during the cell cycle at the present time.

1.8.4. DNA Replication and Synthesis

The early work in this field was reported in 1970, by Burzio & Koide, who found that the treatment of isolated rat liver nuclei with NAD markedly decreased the incorporation of ^{3}H-labelled deoxyribonucleotides into acid-insoluble material. The decrease was ascribed directly to poly(ADP-ribose) formation based on kinetic analysis. This observation was confirmed in other laboratories (Ueda, 1971; Hilz et al., 1971) and extended to different types of cells such as Physarum polycephalum (Brightwell & Shall, 1971), regenerating rat liver (Burzio & Koide, 1972), Ehrlich carcinoma, and HeLa S_3 cells (Hilz et al., 1971), but it was also found that no such phenomenon could be observed in the nuclei of lymphoid cells (Lehmann & Shall, 1972), hepatoma cells (Burzio & Koide, 1972; Yamada & Sugimura, 1973), and adult human fibroblasts (Roberts & Smulson, 1974). Surprisingly the opposite effect, that is, the activation of DNA synthesis was demonstrated in HeLa cells (Roberts & Smulson, 1973; Roberts et al., 1973). Thus, the effect of poly ADP-ribosylation on nuclear DNA synthesis is apparently dependent on the type of cell involved. In the cell nuclei in which NAD inhibition of DNA synthesis occurs, the following mechanisms have been implicated: inhibition of DNA fragmentation (Yamada et al., 1973; Burzio & Koide, 1973), inhibition of template activation

(Burzio & Koide, 1973), release of DNA polymerase from the nuclei (Yoshihara & Koide, 1973), and direct inhibition of DNA polymerase (Nagao et al., 1972). If the increase in the template-primer activity of chromatin, usually observed during the incubation of nuclei, is a consequence of partial degradation of DNA (Roberts et al., 1973; Burzio & Koide, 1971 & 1973), the inhibition of the latter by poly ADP-ribosylation should lead to a lower template-primer activity. This view is supported by the properties of two deoxyribonucleases: Ca^{2+}, Mg^{2}-dependent alkaline endonuclease (Yoshihara et al., 1974) and Mg^{2+} (or Mn^{2+})-dependent exonuclease (Yamada et al., 1974 & 1975). The exonuclease was partially purified from rat liver and shown to be inhibited fairly specifically by poly(ADP-ribose) (Yamada et al., 1975). The endonuclease, originally described by Burgone and co-workers (Hewish & Burgoyne, 1973; Burgoyne et al., 1970), was shown by Yoshihara and co-workers to be ADP-ribosylated; the ADP-ribosylation of the enzyme is accompanied by inactivation (Yoshihara et al., 1975 & 1974). Partial restoration of enzymatic activity on removal of ADP-ribosyl groups by a mild alkaline treatment was also demonstrated (Yoshihara et al., 1974). These results suggest that the modification of this endonuclease is the main molecular basis for the NAD-induced inhibition of DNA synthesis. In further support of this view, the nuclei

of ascites hepatoma AH-130 (in which DNA synthesis is not inhibited or activated by NAD) do not undergo any fragmentation of DNA upon incubation with or without NAD (Yamada et al., 1975; Burgoyne et al., 1970).

In contrast to any of the above results, Roberts et al. (1973; 1974) showed a significant enhancement of DNA polymerase activity as a result of ADP-ribosylation in HeLa cells. The stimulation was specific for formation of poly(ADP-ribose) and varied through the cell cycle. The release of template restriction was proportioned to the capacity of a given cell to synthesize poly(ADP-ribose). The ability of poly(ADP-ribose) to stimulate DNA synthesis was found to be inversely proportional to template restriction throughout the growth cycle of HeLa cells.

Janakadevi (1978) has shown that removal of lysine-rich histones or treatment with heparin increases DNA synthesis in isolated nuclei from pig aorta. The decrease in poly(ADP-ribose) synthesis observed with lysine-rich histone is explained by co-extraction of ADP-ribose transferase and they conclude that removal of lysine-rich histones or ADP-ribose transferase exposes initiation sites for DNA synthesis. Tanigawa et al. (1978) showed that preincubation of isolated nuclei from chick-embryo or hen liver with NAD^+ increased and decreased dTTP incorporation into DNA, respectively. Extraction of NAD-treated embryonic

and adult liver nuclei with 0.35M-NaCl and subsequent reconstitution showed that the factors responsible for suppression or stimulation of DNA synthesis were present in the 0.35M-NaCl extract. The same group later showed that the stimulation of DNA synthesis observed in chick-embryo liver nuclei was due to increased accessibility of the DNA to nuclease (Tanigawa et al., 1978). Subsequently, it was shown by Kitamura et al. (1979) that adminstration of glucocorticoid hormone to chick-embryos caused a decrease in both DNA and poly (ADP-ribose) synthesis. This may be due to loss of enzyme activity as a result of increased nuclear fragility.

Further observations were made by Ghani & Hollenberg (1978) who demonstrated that chick embryo heart cells exhibited higher ADP-ribose transferase activity in isolated nuclei from cells grown in 5% (v/v) O_2 than from cells grown in 20% O_2. They also showed an increased amount of poly(ADP-ribose) in vivo in cells grown in 20% (v/v) O_2. They suggested that in rapidly dividing cells (those grown in 5% (v/v) O_2) the redox potential shifts ($NAD^+ \longrightarrow$ NADH) causing a decrease in poly(ADP-ribose) synthesis and increasing DNA synthesis (Ghani & Hollenberg, 1978). These findings are the result of in vitro studies with isolated nuclei, which have serious limitations in studying the function of poly(ADP-ribose). The use of in vivo systems seem to be

more promising, however the technical problems are enormous. It has been suggested by Berger et al. (1978) that permeabilised cells are better models than isolated nuclei for studying the relationship of poly(ADP-ribose) to DNA synthesis. The latter workers have also found that incorporation of [^{3}H]dTTP into acid-insoluble material does represent semi-conservative DNA replication in permeabilised cells (Berger & Johnson, 1976). It was then concluded (Berger et al., 1978) that inhibition of ADP-ribose transferase had no effect on DNA synthesis and inhibitors of DNA synthesis had no effect on poly(ADP-ribose) synthesis in permeabilised L-cells. They also demonstrated that when simultaneous synthesis of both polynucleotides occurred, the synthesis of one did not effect the other. In addition they found that the rate of synthesis of poly(ADP-ribose) was greater in stationary-phase cells than in exponentially growing cells whereas that of DNA synthesis was highest in exponentially growing cells. However the total ADP-ribose transferase activity did not vary during the growth cycle when the enzyme was measured in the presence of deoxyribonuclease 1 and Triton X-100. This finding indicates that the physiological activity of the enzyme varies, possibly as a result of differences in availability of endogenous acceptors, while the total amount of enzyme remains constant. Similarly Berger et al (1978) were

able to show that decreased DNA synthesis in cells subjected to acute glucose deficiency, vaccinia virus infection and cytosine arabinoside treatment, resulted in increased ADP-ribose transferase activity in permeabilised cells. These studies showed that the greatest increase in ADP-ribose synthesis occurred on cessation of DNA synthesis. However, a transient increase in poly(ADP-ribose) synthesis is seen in cells actively synthesising DNA, in agreement with the results of Lehmann et al. (1974). The relationship between DNA synthesis and poly(ADP-ribose) synthesis has also been shown by Kidwell & Mage (1976) who found that in synchronised cells, poly (ADP-ribose) synthesis occurred mainly as the cells passed through S into G_2. When normal and chronic lymphocytic leukaemia (CLL) lymphocytes were examined with respect to photohaemagglutinin (PHA) stimulation, differences were also observed (Berger et al., 1978). In both normal and CLL lymphocytes PHA stimulation caused increased ADP-ribosylation. The normal cells showed the expected response of DNA synthesis to PHA stimulation, but the response of DNA synthesis in the CLL cells was decreased and much delayed. It was suggested that this may be due to either damaged or disordered DNA or the presence of immature differentiating lymphocytes (Berger et al., 1978).

These results are contrary to those obtained in L cells with respect to the relationship between DNA and poly (ADP-ribose) synthesis. Thus, permeabilised cell systems have proved very useful for the analysis of poly(ADP-ribose) and its relation to nuclear function. However it seems that the relationship may only be unequivocally proven when true *in vivo* systems are used. However at present the technical problems inherent in such studies are insurmountable.

1.8.5. DNA Transcription

In 1969, Haines *et al.*, observed a higher activity of ADP-ribose transferase in differentiated cells engaged in RNA synthesis compared to cells engaged primarily in DNA synthesis (Haines *et al.*, 1969). However the formation of poly(ADP-ribose) in isolated liver nuclei did not change their capacity to synthesise RNA (Burzio & Koide, 1971; Roberts *et al.*, 1973). Muller *et al.* (1974) observed that in oestrogen-stimulated immature quail oviduct progesterone-induced gene expression (including avidin synthesis) was accompanied by a considerable reduction on ADP-ribose transferase activity and an increase in RNA polymerase I and II activities (Muller *et al.*, 1974). In contrast, Hilz & Kittler (1971) showed that there was no change in ADP-ribose transferase activity in liver of sham-

operated, adrenalectomized or cortisol-treated rats, suggesting ADP-ribosylation played no role in DNA transcription (Hilz & Kittler, 1971). It has been shown by Tsopanakis et al. (1978) that the specific activity of ADP-ribose transferase in nuclei and nucleoli of Tetrahymena is the same even though nucleoli exhibit high ribosomal transcriptional activity. This is independent of the presence or absence of RNA synthesis (Tsopanakis et al., 1978). A positive association of ADP-ribose transferase activity with transcription has been suggested from the studies of Smulson et al (1977). They fractionated sonicated HeLa-cell chromatin by using ECTHAM-cellulose chromatography and glycerol-gradient centrifugation in an attempt to separate transcriptionally active and inactive chromatin (Mullins et al., 1977). Smulson concluded that ADP-ribose transferase activity is primarily associated with extended and transcriptionally active chromatin; the transcriptionally inactive, condensed, chromatin fractions contained relatively low ADP-ribose transferase activity (Mullins et al., 1977). However, Yukioka et al. (1978) pointed out that mechanical shearing as used by Mullins et al. (1977) did not adequately separate active and inactive regions of chromatin structure. Rat liver chromatin has been fractionated using selective shearing with deoxyribonuclease II followed by perferential precipitation.

This has been shown to fractionate chromatin into transcriptionally active and inactive fractions (Gottesfeld et al., 1974, 1975). Yukioka et al. (1978), then showed that ADP-ribose transferase activity is not preferentially localised in transcriptionally active chromatin regions using Gottesfelds method. Furneaux & Pearson (1980) examined a number of events relating to ADP-ribose metabolism during serum-stimulated growth of baby hamster kidney (BHK)-21/C_3 fibroblast. They supported the hypothesis that the intracellular content of NAD^+ is correlated with the growth-state of the cell, and they also reported the association of high intracellular NAD^+ concentration with increased cellular proliferation. In their study, they suggested that the activity of RNA polymerase I may be regulated by ADP-ribosylation of the enzyme itself, or of an associated sub-unit that influences enzyme activity (Furneaux & Pearson, 1980). Finally the protein A_{24}, a repressor of ribosomal gene activity (Ballal & Busch, 1973) was found to be ADP-ribosylated (Okayama & Hayaishi, 1978). This gave an indirect indication of involvement of poly(ADP-ribose) with DNA transcription.

1.8.6. DNA Repair

DNA repair is a dynamic and continuous process which occurs in all cells. It therefore could be a likely target for modulation by ADP-ribosylation of involved

proteins. Miller et al. (1975) observed that DNase I or micrococal nuclease increases formation of poly(ADP-ribose) in HeLa nuclei and that fragmented DNA stimulates partially purified ADP-ribose transferase better than native DNA (Roberts et al., 1975). A direct function of poly(ADP-ribose) in DNA has been postulated by Gill (1975) on the basis that fragmented DNA is a better activator of ADP-ribose transferase than native DNA in vitro. The enzyme also appears to have a higher activity in radiosensitive tissues (Gill, 1975). The nucleases can vary in their effect from stimulation to inhibition together with alterations in either the chain number or the chain length or both (see references below). These different responses seem to depend on a number of conditions such as the NAD concentration, the method of nuclear preparation, the nuclease treatment procedures and the inhibitors used (Nishizuka et al., 1968; Doly,& Mandel 1967; Reeder et al., 1967; Fujimura et al., 1967 and Janakidevi et al., 1974).

Whish et al. (1975) and Smulson et al. (1975) have studied the effect of damaging DNA chemically on the synthesis of poly(ADP-ribose). They showed that the ADP-ribose transferase activity of nuclei of Physarum polycephalum and HeLa cells, respectively, was stimulated after pretreatment with streptozotocin, the 2-deoxy-D-glucose derivative of the alkylating agent,

N-methyl-N-nitrosourea. Jacobson (1978) examined the effect on NAD^+ concentrations of, N-nitroso compounds which were direct-acting, indirect-acting or non-carcinogens, in both 3T3 cells and mitogen-stimulated human lymphocytes. Direct-acting carcinogens caused large decreases in NAD^+ in both 3T3 cells and lymphocytes. Indirect-acting agents caused a decrease in NAD in lymphocytes but not in 3T3 cells and non-carcinogens did not effect the NAD^+ content of either cell type (Jacobson & Jacobson, 1978).

Sudhakar et al. (1979) showed that the N-methyl-N-nitrosourea-induced increase in ADP-ribose transferase at the nucleosome level was a result of increased availability of protein acceptors. The same group (Sudhakar et al., 1979) compared alkylation of chromatin by N-methyl-N-nitrosourea (MNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). In contrast with N-methyl-N-nitrosourea, treatment of HeLa cells with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea resulted in a slight decrease in ADP-ribose transferase activity in isolated nuclei. MNU primarily causes DNA strand breakage whereas CCNU causes cross-linking. In contrast to MNU, CCNU caused a slight decrease in ADP-ribose transferase activity. They were able to show by nuclease digestion, that the differential effects of the two compounds on ADP-ribose transferase may be explained by the site of

alkylation of the two compounds. MNU preferentially alkylates internucleosomal regions of DNA, suggested as the sites of transferase activity (Mullins et al., 1977), whereas CCNU alkylates the core particles since MNU and CCNU may also carbamoylate proteins, the preferential modification of non-histone proteins by CCNU may have a direct effect on the transferase itself. It has been also shown by Davies et al. (1976, 1977) that poly(ADP-ribose) levels increased on treatment of nuclei with the polypeptide antiboitic neocarzinostatin (Davies et al., 1976, 1977). Berger et al. (1979) demonstrated that poly(ADP-ribose) synthesis was increased in permeabilised cells pretreated with U.V. irradiation. They also showed that the DNA synthesis observed after irradiation consisted of repair synthesis and not semi-conservative replication (Berger et al., 1979).

It has been observed that known inhibitors of DNA repair such as theophylline and caffeine (Cleaver & Thomas, 1969; Lehmann & Kirk-Bell, 1972) are also inhibitors of ADP-ribose transferase in isolated nuclei (Levi et al., 1978). The effect of other inhibitors of ADP-ribose transferase on the ability of cells to repair DNA damage in vivo and their subsequent survival was studied by Smulson et al. (1977). They showed that mice injected with L1210 tumour cells exhibited an increased survival over controls in the presence of MNU and that

co-administration of nicotinamide increased this survival to an even greater extent. Shall et al. (1977) and Nduka et al. (1980) substantiated this apparent enhancement of cytoxicity of DNA-damaging reagents by inhibitors of ADP-ribose transferase and they showed that the effect was synergistic. Durkacz et al. (1980) examined the relationship between ADP-ribose transferase activity, cell survival and excision repair in conjunction with inhibitors of the enzyme. By using permeabilised mouse L1210 cells, they showed that specific inhibitors prevented rejoining of DNA strand breaks caused by dimethyl sulphate (DMS)and as a result, cytotoxicity was enhanced. However inhibitors might also inhibit the DNA repair itself. To check this Durkacz et al (1980) depleted cells of NAD^+ by growing in nicotinamide-free medium. The rejoining of strand breaks was shown to be prevented under these conditions, using alkaline sucrose gradients (Durkacz et al., 1980). Jacobson & Narasimham (1979) also demonstrated this phenomenon using 3T3 cells.

These findings suggested that ADP-ribosylation of nuclear protein may play a role in DNA repair.

Farzaneh et al. (1980) showed that in chick myoblasts the transition from the proliferating to the differentiating state was associated with DNA strand breakage and an increase in ADP-ribose transferase activity. They also showed that although the expression

of the differentiated state is inhibited by transferase inhibitors, the appearance of DNA strand breaks was not. This finding suggested that the synthesis was involved in the process of differentiation at a point distal to the appearance of DNA strand breaks.

CHAPTER 2

Germination

2.1. Germination in General

Much of the current research on wheat embryos has been carried out by Chen and his coworkers. They have reported that water imbibition by the dry wheat embryo triggers an array of molecular changes which causes the transfer of the embryo from a latent stage into an active development stage, representing an important phase in the life cycle of the plant (Chen et al., 1968). The ungerminated embryo is able to withstand extremes of environmental stress, and the genome of the ungerminated embryo is preserved in an active form. The DNA is degraded if any water stress occurs after germination. DNA damage is always seen if water stress (water deprivation) occurs within 18 hours of addition of water (Mory et al., 1972).

2.2. Biochemical Development During Germination

2.2.1. Protein Synthesis in Germinating Wheat Embryos

Marcus et al. (1966) studied the onset of protein synthesis in germinating wheat embryos and suggested that it occurred in two phases: the first phase occurred directly when water was added to the embryos, and lasted for about half an hour. This phase was apparently controlled

by an activation of a ribose-messenger system or, more specifically, by the formation of functional polyribosomes. This initial phase of protein synthesis was observed qualitatively by assays *in vivo* but was best studied quantitatively by *in vitro* assay of amino acid incorporation with isolated ribosomes in a cell free protein synthesis system.

The second phase of protein synthesis occurred between half an hour and sixteen hours of germination and was superficially similar to phase one. It was accompanied by an increase in the *in vitro* activity of isolated ribosomes and a simultaneous increase in "polyribose" content, the overall effect being a much enhanced capacity for protein synthesis in phase two. Thus Marcus *et al*. (1966) concluded that ribosomes isolated from dry embryos, in contrast to those isolated from embryos germinated for 16 hours, are capable of only a low level of amino acid incorporation into protein. They also concluded that polyribosome formation might be the factor limiting protein synthesis in the early stage of germination (first phase of protein synthesis). Weeks & Marcus (1971) also found that during early germination the preformed "messenger fraction" found in dry embryo, disappeared and was apparently incorporated into polyribosomes. The regulation of this process was suggested to be via an ATP-sensitive system. In support

of this Marcus et al (1966) observed that the formation of polyribosomes can be achieved in an in vitro experiment by incubation of extracts of ungerminated wheat embryos provided that such extracts were supplemented with ATP. This suggested that the ATP level may be a limiting component in polyribosome formation in vivo. Therefore ATP may be a primary regulatory factor in the germination of wheat embryos .

Subsequently Marcus (1974) found that the endogenous level of ATP increased markedly during the first hour of germination with only small changes during the next 15 hours. (Obendorf & Marcus, 1974).

2.2.2. Nucleic acids synthesis in germinating wheat embryos

It has been suggested by Chen (1973) that protein synthesis in the G1 phase of the cell division cycle mediated the initiation of DNA replication in germinating wheat embryos. Chen also studied the sequential activation of the genome by analysing tRNA, rRNA and mRNA transcription during germination, and this led him to divide early germination into three phases.

Phase 1: occurs between 0 and 9 hours of germination. If protein synthesis is blocked in this period, a complete inhibition of DNA synthesis occurs.

Phase II: This occurs between 9 and 15 hours of germination. Here there appears to be a semiquantitative

relationship between the time span of protein synthesis and the rate of DNA synthesis.

Phase III: This occurs at 18 hours after germination, when DNA replication is independent of protein synthesis.

To explain the apparent relationship between the blocking of protein synthesis of early wheat embryos germination and the initiation and replication of DNA (Phase I), Mory et al. (1972) suggested that the germinating wheat embryo does not enter the DNA synthesis phase before 8 hours of germination and that furthermore DNA synthesis may be dependent on the synthesis of certain proteins produced in G1. When early protein synthesis was blocked, using blasticidin S (a specific inhibitor of protein synthesis), they found that the germinating wheat embryo did not enter the DNA synthetic phase. This supported the earlier findings that protein synthesis plays a major role in the mechanism that controls the switching on of cellular DNA replication. Mory et al. (1972) has thus proposed the following steps which control DNA synthesis:

1. unavailability of an active DNA template.
2. Shortage of enzymes involved in the metabolism of DNA precursors and resulting shortage of DNA precursors.
3. Shortage of enzymes directly involved in the polymerisation of DNA such as DNA-dependent DNA polymerase.
4. Presence of inhibitors of one or more of the mentioned enzymes.

Chen & Osborne (1970) have found that DNA isolated from germinated embryos differs from that isolated from ungerminated embryos, suggesting that modification of the DNA is occurring in early germination. The enzymes connected with DNA synthesis in higher plants appear to have very similar properties to those found in animal and bacterial systems. Several of these enzymes have been studied in detail by Harland et al. (1973) in synchronous plant cell cultures. An overall view of nucleic acid metabolism in higher plants can be found in the book by Bryant (1976).

2.2.3. Histone studies in plants compared to those in animals.

Chromatin contains both basic (histone) and acidic (non histone) proteins, which show very significant differences in physical and chemical properties. Both types of protein play an important and central role in chromatin structure and function.

Classically, histones are prepared by extraction from isolated chromatin (or nuclei) with dilute mineral acid (Bonner et al., 1968).

It has been demonstrated in recent years that great similarities exist between certain plant and animal histones (Nadeau et al., 1977). Comparisons of the amino acid sequences of the arginine-rich histones

H3 and H4 from the pea bud and calf thymus, have indeed shown a remarkable conservation of their primary sequences. In these two organisms, only 2 of the 102 amino acid residues differ in H4 (Delange et al., 1969) and 4 out of 135 residues are different in the case of H3 (Patthy et al., 1973). Furthermore, in all instances the amino acid replacements are conservative.

On the other hand, the very lysine-rich histone H1 shows considerable variation even in rather closely related species. Many such examples are known (Panyim et al., 1971; Sherod et al., 1974; Bustin & Cole, 1968) from the extensive comparative investigations carried out on vertebrate histones. When invertebrate (Tessier & Pallotta, 1973; Alfageme et al., 1974) and protozoan H1 histones (Hamano & Iwai, 1971; Gorovsky et al., 1974; Caplan, 1975) were compared with each other and with the corresponding vertebrate fractions, many differences were found. In general, available data on plant histones indicate that the H1 fraction has a slower migration on acrylamide gels (Sommer & Chalkey, 1974; Nadeau et al., 1974; Spiker, 1975), a more complex electrophoretic pattern (two or more subfractions) and higher molecular weight (up to 3,000more) than its counterparts in animal cells. Among vertebrates, the slightly lysine-rich histones (H2A and H2B) display only minor differences in their respective electrophoretic behaviours (Panyim

et al., 1971). Although the order of migration may vary, these fractions are always located between reduced H3 and H4 histones in the electrophoretic system of Panyim & Chalkley (1969). The same position was found for the invertebrate H2A and H2B fractions thus far examined (Tessier & Pollotta, 1973; Alfageme et al., 1974; McMaster-Kaye & Kaye, 1973). In higher plants, however, no histones migrate between fraction H3 and H4 (Sommer & Chalkley, 1974; Nadeau et al., 1974; Spiker, 1975; Brandt & Von Holt, 1975). Instead, histones are found between H1 and H3, a situation which generally does not exist in vertebrates. The molecular weights of these plant histones are observed to range from 15000 daltons to 17000 daltons (Sommer & Chalkley, 1974 and Nadeau et al., 1974) and to have substantially higher values than those of H2A (12500 daltons) and H2B (14000 daltons) vertebrate fractions. These plant histones have been called PH_I, PH_{II} (Nadeau et al., 1974) F2b, F2a (Sommer & Chalkley, 1974) or IIa IIb (Fambrough & Bonner, 1966).

In other comparative work, Spiker (1975) studied the electrophoretic pattern of histones from several plants ranging from the primitive moss Polytrichum to highly evolved Liliaceae. He noted the identical migration of fractions H3 and H4 from calf thymus and from the various plant species studied. He also reported the presence of plant-specific bands between H1 and H3.

Although a 'H2A-like' histone was observed in all species studied, the 'H2B-like' histone seemed to be absent from primitive plants and to appear first in ferns. The more evolved plant species thus seem to possess three fractions, H1, H2B (PH_I) and H2A (PH_{II}), which differ from animal histones by their higher molecular weights and their slower mobilities on polyacrylamide gel electrophoresis. Such differences are evidently related to the primary structure of these proteins.

Rye histones might each consist of an animal histone plus some 20-30 amino acids, or the sequence of these proteins might be partially or totally different from those of their animal counterparts. To choose between these possibilities Nadeau et al. (1977) analysed the amino acid compositions and the tryptic fingerprint maps of the rye and thymus H1, H2B (PH_I) and H2A (PH_{II}) histones. From the data obtained from the fingerprints, as well as those from amino acid composition analyses (see Nadeau et al., 1977), they concluded that the calf and rye histone fractions H1, H2B (PH_I) and H2A (PH_{II}) have different primary structures. They also exclude the possibility that the plant histones consist essentially of an animal protein plus a segment of 20-30 amino acids. Although it is difficult to evaluate the difference between the fractions without knowing the

exact sequences, the tryptic fingerprints differ to such an extent that major changes in the primary structures can be expected.

A good correspondence in amino acid composition for both pea and rye H2B (PH_I) and H2A (PH_{II}) histones was observed when these results were compared with those of Fambrough et al. (1968). Thus one can conclude that while histones are found in plants they are markedly different from non-plant histones. Such a situation makes the analysis of histone ADP-ribosylation in wheat embryo a somewhat complicated process (see Section 2.4.).

2.2.4. Adenosine Triphosphate Content and Germination

The need for energy to initiate and maintain growth activities is universal (Ching, 1972). Aging stress which is produced during a long period of storage, was found to reduce the ATP content in imbibed seeds (Ching, 1973). Killing seeds by using high temperature at high moisture content also decreased ATP content from an average of 11 to 2 pmoles per ryegrass seed and from 150 to 25 pmoles per rape seed. The level of ATP in plant tissue appears to be a very sensitive index of environmental and developmental changes. It is rapidly reduced in cotton seedlings when they are chilled (Stewart & Guinn, 1969), under anaerobiosis in imbibed lettuce seeds (Pradet et al., 1968) and in pea roots

exposed to saline media (Hasson-Porath & Paljakoff-Mayber, 1971). ATP levels increase in content with the synthesis of organelles and enzymes in non-dividing tissues (Ching & Ching, 1972; Pradet et al., 1968) and also rise in step with cell numbers and the size of growing organs (Ching & Kronstad, 1972).

The isolated wheat embryo was ideally suited for demonstrating the early change in ATP because of its rapid rate of imbibition and its presumably concomitant accelerated metabolism (Obendorf & Marcus, 1974).

Alternative to ATP concentration per se, there are at least two additional possible regulatory mechanisms that would involve the adenine nucleotide. The first of these, suggested by Freudenberg & Mager (1971) was an inhibition of protein synthesis by high levels of ADP and AMP. Activation of protein synthesis, according to this concept, would involve the reduction of the levels of these nucleotides. Consistent with this type of regulation is the data for wheat embryo (see Obendorf & Marcus, 1974) which showed that both ADP and AMP decrease considerably during the period between 1 and 6.5 hours of germination.

The alternative type of regulation, advanced by Chapman et al. (1971) considers the adenylate energy charge as the primary regulator and allows that growth will occur only at energy charge values above about 0.7.

The observation with wheat embryos that the period preceeding rapid growth was marked by an increase in adenylate charge from approximately 0.6 to above 0.8 (see Obendorf & Marcus, 1974) was also consistent with this conclusion and is in accord with results for several other seed species (Ching, 1972; Ching & Ching, 1972; Ching & Kronstad, 1972).

Based on these considerations, it appears likely that the adenine nucleotides are a major component in the regulation of early embryo germination. Obendorf & Marcus (1974) suggested that the initial activation of protein synthesis is closely linked to the increased ATP level and that the change in adenylate charge is more related to the quiescent period required for subsequent embryo growth. This latter period has previously been defined as a unique 'germination phase' (Marcus, 1969).

Many workers have therefore shown that ATP levels alter quite drastically during germination. This must have important implications for NAD synthesis (see section 2.3) since the latter is dependent on ATP as a substrate for its synthesis. Furthermore, one might expect protein ADP-ribosylation to be affected by these variations in ATP levels.

2.3. NAD Metabolism in Plants

Several workers have investigated the changes in levels of nucleotides and nucleosides during germination. It has been shown (Brown, 1965, 1967) that during the first 40 hours of germination of pea, the AMP and ADP levels fell, whilst the ATP level initially rose and then fell. Obendorf & Marcus (1974) have shown that there was a 10 fold increase in the ATP level of germinating wheat embryos within one hour of the onset of germination.

The NAD content of various cereals has been examined in germinating wheat and oats (Bevilacqua & Scotti, 1953; Bevilacqua, 1955) and in germinating rice seeds (Mukherji et al., 1968). It was shown that the overall NAD level increased rapidly. However, the rate of turnover was not measured. Reed (1970) has shown by using germinating peanuts that at the onset of germination there was a significant conversion of NAD to NADP with a coincident rise in NAD kinase, suggesting a possible active role for these compounds during germination.

The metabolism of NAD has also been studied in normal wheat leaves (Godavari & Waygood, 1970), in bean leaves (Chen et al., 1974) and in puccina infected wheat leaves (Pao et al., 1974). Leienbach et al.,(1976) have reported in a series of papers on the metabolism of

nicotinic acid, nicotinamide and their derivatives in continuous plant cell cultures (Heeger et al. 1976; Leienbach et al., 1976; Neuhann et al., 1979). They have shown that NAD metabolism was via the Preiss-Handler cycle (see Figure 2.1.) (Preiss & Handler, 1958) but that one of two derivatives of nicotinic acid (N methyl nicotinamide and N-α-L-arabino-nicotinamide) acts as a reservoir for nicotinic acid (and therefore NAD) depending upon the species of cell used. Cultures of legume cells (e.g. mung bean, soyaboan and garbarrzo bean) accumulated N-methyl nicotinamide, and umbelliferae cells (e.g. parsley) accumulated N-α-L-arabino-nicotinamide. The same group have also shown that the adenine of NAD was degraded in cell cultures via hypoxanthine, xanthin, allantoin and allantoic acid with an accumulation of the latter two compounds.

Sufficient is therefore known about NAD biosynthesis in plants to allow meaningfull studies into the next step of NAD metabolism, That is, ADP ribosylation of nuclear proteins (Section 2.4.).

2.4. Poly(ADP-ribose) in Plants

There are several reports which deal with the occurrence of poly(ADP-ribose) in plants such as in

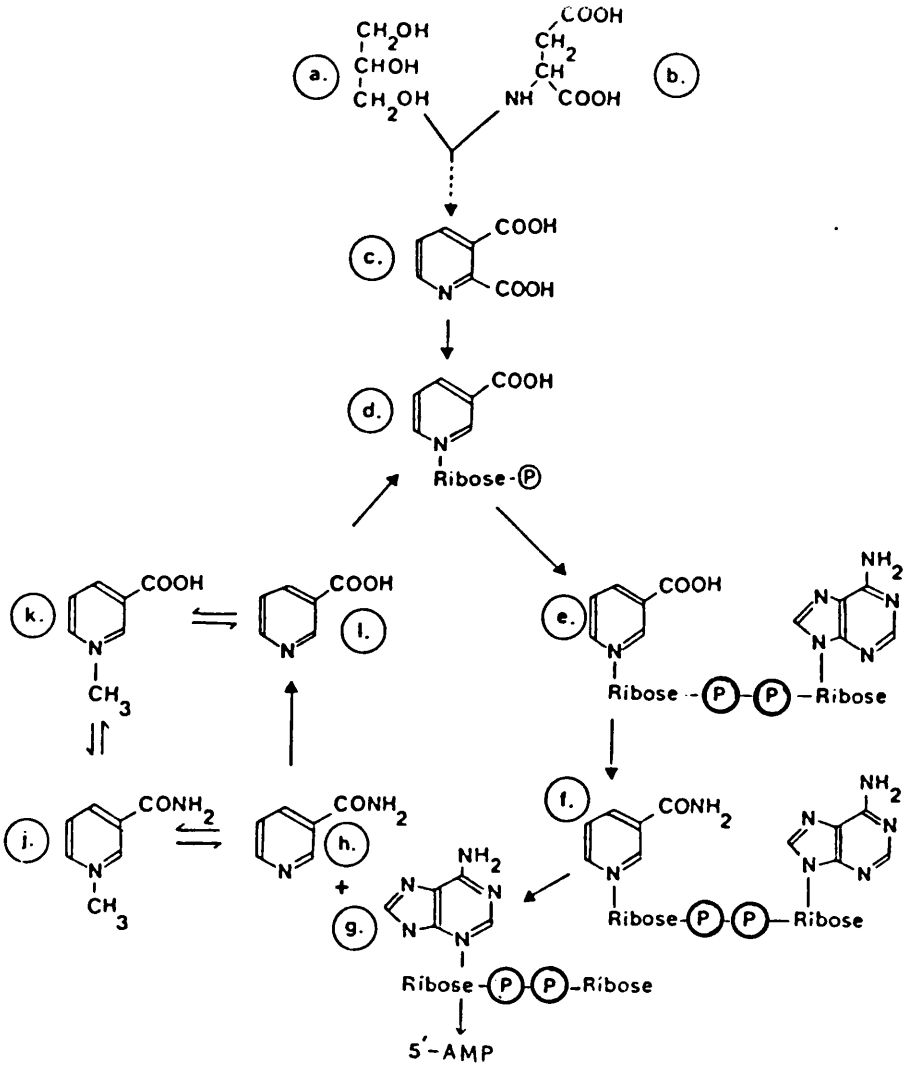


Figure (2.1) NAD metabolism in plants: the Preiss-Handler Pathway.

a Glycerol
b Aspartic acid
c Quinolinic acid
d Nicotinic acid mononucleotide
e Nicotinic acid adenine dinucleotide
f Nicotinamide adenine dinucleotide (NAD)
g Adenosine diphosphate ribose (ADPR)
h Nicotinamide
j N-methyl nicotinamide
k N-methyl nicotinic acid
l Nicotinic acid

meristematic (root meristem) or 'quiescent' (seed embryo) plant tissue (Payne & Bal, 1976); wheat germs (Whitby & Whish, 1977); plant tissues culture cells (Willmitzer, 1979) and rye (Laroche et al., 1980). The covalent incorporation of NAD into chromosomal proteins had only been observed in nuclei or chromatin from animal tissues. There was no evidence that this modification could also occur in the plant kingdom (Stocken, 1978). This may be a corrollary of the great difficulties in the isolation of highly purified and physiologically active nuclei from plant tissue. Willmitzer (1979) described the covalent modification of chromosomal proteins by poly(ADP-ribose) in plant nuclei obtained from transformed tissue cultures of Nicotiana tabacum. He showed a striking similarity to the animal system previously described e.g. the action of several inhibitors, the average chain length, the fact that >80% of the poly(ADP-ribose) is covalently bound to protein acceptors, the nature of this covalent linkage as based on its instability in neutral hydroxylamine and even the main acceptors among the histone species i.e. H1 and H2A/H2B.

The inhibitory action of deoxythymidine (3mM) or nicotinamide (3mM) in plants(Willmitzer, 1979) exhibits a strong resemblance to animal systems where thymidine derivatives as well as nicotinamide were shown to inhibit the poly ADP-ribosylating activity (Preiss et al., 1971). Furthermore

this inhibition demonstrated that the formation of trichloroacetic acid-precipitable products was an enzymatic reaction and not a chemical reaction. This has been discussed as a possible pitfall in animal systems (Kun et al., 1976).

Thus it is clear that the ADP-ribosylation of plant nuclear proteins has not been studied in the same detail as that of non-plant systems, possibly because of the enormous technical difficulty of working with plant material.

2.5. ADP-Ribosylation of Histones in Plants

The presence of poly(ADP-ribose) in wheat and rye histones has been established by enzymatic digestion, polyacrylamide gel electrophoresis and column chromatography (Whitby et al., 1979; Laroche et al., 1980). These groups have investigated the modification of histones by ADP-ribose transferase. They found both by polyacrylamide gel electrophoresis and by column chromatography, that the core histone H2B was extensively modified and that histone H1 and histone H2A were modified to a smaller extent. These findings were quite different from those originating from some animal cells where histone H1 was the major acceptor (Hayaishi et al., 1979; Hilz & Stone, 1976) and where histones H3, H2B and H2A were often modified to a lesser extent. These results might suggest a differential

accessibility of core histones and internucleosomal histones to the ADP-ribose transferase in plants. Also implied is a different localisation of the enzyme in the nucleosome structure of rye. These results are in agreement with those of Willmitzer (1979) in that histones H2A and H2B were the main acceptors. The results from the exclusion chromatography are slightly different from the ones obtained by polyacrylamide gel electrophoresis since H1 is more modified as compared with the H1 run on polyacrylamide gel. This might suggest that ADP-ribose or poly(ADP-ribose) attached to histone H1 was more labile under gel electrophoresis than with column chromatography. Possible explanations of this difference are that material always stays on top of the gel or that there are histone aggregates on the top of the gel as described by Adamietz et al. (1979).

2.6. The Effect of Polyamines on ADP-Ribosylation of Histones in Plants

In view of the effect of polyamines and Mg^{++} in stimulating poly(ADP-ribose) synthesis in isolated rat liver nuclei (Tanigawa et al., 1977; Perella & Lea, 1978, 1979) and also the observation that plant tissues contain fluctuating levels of polyamines (Ramakrishna &

Adiga, 1974; Bagni, 1970; Smith & Wilshire, 1975, 1977; Villaneuva & Adlakha, 1978). The effect of polyamines on poly(ADP-ribose) synthesis in isolated wheat nuclei was studied by Whitby *et al.* (1979). Since histones are one of the most studied acceptors of poly(ADP-ribose) (Hilz & Stone, 1976), the effect of polyamines on histone ADP-ribosylation in wheat nuclei has also been investigated in an attempt to gain insight into the possible role of poly(ADP-ribose) and to compare the plant and mammalian systems (Tanigawa *et al.*, 1977; Perella & Lea, 1979). Whitby *et al.* (1979) showed that the polyamines spermine, spermidine and putrescine exhibit a dose dependent effect in increasing the *in vitro* ADP-ribosylation of nuclei isolated from wheat embryos. Mg^{++} also shows a similar but somewhat lesser effect on ADP-ribosylation. These results are similar to observations made by Tankgawa *et al.* (1977) on the effect of Mg^{++} and polyamines on ADP-ribosylation in rat liver nuclei. They also found the stimulation of total poly(ADP-ribose) synthesis was accompanied by a similar increase in the total histone associated poly(ADP-ribose) for Mg^{++} and polyamines. These findings differ from those obtained in rat liver (Tanigawa *et al.*, 1977) and show that in wheat nuclei there is no preferential ribosylation of histones caused by Mg^{++} or polyamines. It was also seen that the distribution of ADP-ribose between H1 and the other

histones was not greatly affected by Mg^{++} or polyamines. This is again different to the situation in rat liver where it has been shown that 1mM spermine causes no change in the extent of ADP-ribosylation of total histones although a redistribution of the ADP-ribose from the core histones to H1 was observed (Perella & Lea, 1978, 1979). However, in view of the observed ADP-ribosylation of these histones in isolated wheat nuclei and the stimulation by polyamines, it may well be that ADP-ribosylation of histones in plant cells is involved in extending or condensing chromatin as has been postulated for mammalian cells (Perella & Lea, 1978, 1979; Lorimer et al., 1977; Byrne et al., 1978; Whitby et al., 1979; Stone et al., 1977).

2.7. Poly(ADP-ribose) Glycohydrolase in Plants

The ADP-ribose transferase activity was determined in plants as previously described (see Section 2.4). The ADP-ribose transferase activity in a crude chromatin preparation from wheat seeds (Whitby & Whish, 1977) was inhibited by either of two inhibitors, nicotinamide or 3-aminobenzamide (Purnell & Whish, 1977). By using 3-aminobenzamide, the presence of poly(ADP-ribose) glycohydrolase was investigated in detail (Whitby & Whish, 1978). The latter group showed that

poly(ADP-ribose) degrading activity was present in the ungerminated embryo. The activity was found to be poly(ADP-ribose) glycohydrolase by showing that the product of poly(ADP-ribose) degradation was ADP-ribose and not 5-phosphoribosyl-5-AMP. The latter would have been expected if degradation has been due to phosphodiesterase activity.

CHAPTER 3

Materials and Methods

3.1. Materials

3.1.1. Wheat Seeds:

Wheat (Triticum Aestivus 1) seeds were obtained from Wiltshire Farmers Ltd. of Melksham, Wiltshire. They were supplied in 50kg sacks, and were pretreated with organic mercury seed dressing. The seeds were stored dry, in the dark, at 0-4°C until used.

3.1.2. Radioactive Materials:

[Methyl-^{3}H] thymidine, 47.5 Ci/mmol, 1 mCi/ml;
[2-^{3}H] adenosine, 24 Ci/mmol and 21 Ci/mmol, 1 mCi/ml;
L[4,5-^{3}H]leucine, 53 Ci/mmol, 1mCi/ml;
[5-^{3}H] uridine, 25.5 Ci/mmol, 1 mCi/ml.
These materials were obtained from the Radiochemical Centre, Amersham.

[^{3}H] NAD was synthesised in this laboratory by Dr. W.J.D. Whish from [^{3}H] ATP using the method of Ohtsu & Nishizuka (1971). It was at least 99% pure as estimated by thin layer chromatography and had a final specific activity of 20 mCi/µmol, 1 mCi/ml in 50% ethanol.

3.1.3. Chromatography Materials:

Aminoethyl cellulose from Sigma.

Polyethyleneimine cellulose (PEI-cellulose) thin layer sheets 20 x 20 cm from J.T. Baker Chemical Co.

Disposable polypropylene columns 6 x 0.5 cm from Wright Scientific Ltd.

3.1.4. Counting Materials:

Toluene from BDH Chemicals Ltd., Triton X-100 from Sigma. 2,5-diphenyloxazole (PPO) from Packard Instrument Co. Glass fibre discs (GF/C) and paper discs from Whatman Ltd.

3.1.5. Inhibitors:

Thymidine from Sigma; 3-aminobenzamide was synthesised as described by Purnell & Whish (1980); cordycepin from Sigma.

3.1.6. Caesium Chloride Density Gradient Centrifugation :

Caesium chloride from Sigma; urea from BDH Chemicals Ltd.

3.1.7. Gel Electrophoresis:

Methylenebisacrylamide from BDH Chemicals Ltd.; acrylamide from Fisons; soluene 350 and hyamine hydroxide from Packard Instrument Co.; N,N,N',N'-tetramethylethelene-diamine (TEMED), β-mercaptoethanol and ammonium persulphate from BDH Chemicals Ltd.

3.1.8. Enzymes:

Spleen phosphodiesterase (from bovine spleen), EC 3.1.4.18 from Sigma. The following enzymes were bought from Sigma: snake venom phosphodiesterase (SVPDE) (from Cortalus adamanteus) EC 3.1.4.1.; alkaline phosphatase (from calf intestinal mucosa) EC 3.1.3.1.; RNase A (from bovine pancreas) EC 3.1.4.22; DNase 1 (from bovine pancreas) EC 3.1.4.5.; finally pronase (from Streptomyces griseus) EC 3.4.21.4 was obtained from Calbiochem.

3.2. Methods

3.2.1. Isolation of Wheat Embryos

Wheat embryos were prepared according to Johnston & Stern (1957) and modified as follows:-

100gm of dry seeds were homogenised in a 5 litre stainless steel Waring blender together with small fragments of dry ice. The homogenised material was placed in standardised brass sieves, in a series of decreasing mesh sizes, 1.70mm, 1.18mm and 600µm respectively. The embryos together with starch grains and chaff were isolated on the 600µm sieve. Material which would not pass through the 1.7mm sieve was re-homogenised and re-sieved to obtain as great a yield of embryos as possible.

The crude embryos from the 600μm sieve were then added to a cyclohexane: carbon tetrachloride density mixture (10:25 (v/v)). The embryos which floated on the surface were skimmed off using a tea strainer and air dried. Any chaff which remained was blown off by a fast jet of air. Finally, the embryos were stored dessicated at 4°C under vacuum in the dark until needed.

The embryos' viability was tested regularly in two ways:-

a) embryos were germinated in the dark at 26°C in a petri dish containing 3 filter papers (7cm Whatman No. 1) and 6mls of germinating medium (1% (w/v) glucose and 0.01% (w/v) streptomycin sulphate (SM)).

b) the embryos were placed on agar dishes (1% (w/v) Oxoid No. 1, 1% (w/v) glucose and 0.01% streptomycin sulphate), and incubated at 26°C in the dark for 24 hours.

Germination was considered to have occurred when the root hairs first become visible.

3.2.2. Preparation of Nuclei from Viable Embryos

The whole preparation was carried out at 0-4°C.

0.5gm of embryos were homogenised in 15mls of nuclei isolation buffer (0.1M triethanolamine (TEA) - HCl pH 8.2), using either a hand held potter (PTFE) homogeniser or a glass homogeniser. The homogenate was filtered through

one thickness of muslin and centrifuged in an MSE bench centrifuge at 110g for 2 minutes. After discarding the precipitate, the supernatant was re-centrifuged in a bench centrifuge at 2,700g for 15 minutes. The pellet which contained nuclei was made up to 1ml, using nuclei isolation buffer and sonicated for 4 seconds using an ultra rapidas sonicater. This preparation was used routinely.

3.2.3. Scintillation Counting

Two different scintillants were employed in these studies:

a) for non-aqueous samples, a scintillant composed of 0.5% (w/v) 2,5'-diphenyloxazole (PPO) and toluene was used;

b) for aqueous samples, the scintillant was composed of 0.5% (w/v) PPO, 30% (w/v) Triton X-100 and 70% (v/v) redistilled toluene. This scintillant would accept up to 10% (v/v) aqueous material. Usually 2.0ml of scintillant was used except in some aqueous samples containing low radioactivity, which required larger samples for accurate counting. The paper disc (see Section 3.2.4.) was placed on the base of the scintillation vials (sample face upwards). The radioactivity was determined by scintillation counting in 2ml 0.5% (w/v) PPO in toluene using a Packard Tricarb

scintillation counter.

3.2.4. Estimation of Acid Insoluble Radioactivity

Two different methods for estimation of the acid insoluble radioactivity were generally used. The first one was direct acid precipitation. A 200µl aliquot of sample containing the acid insoluble radioactivity was added to 2mls of 20% (w/v) trichloroacetic acid (TCA) and placed on ice for 30 minutes. The mixture was filtered onto a Whatman (GF/C) disc, which was washed 4 times with 20% (w/v) TCA and finally with about 10ml of absolute ethanol. The discs were dried in an oven at 70°C for 15 minutes before counting (See Section 3.2.3.)

The second method involved the use of 2.4cm diameter Whatman No. 1 filter paper discs which had been pretreated with TCA. These were placed in 20% (w/v) TCA in diethyl-ether for 10 minutes and air dried. Samples of up to 50µl were placed onto a disc and allowed to dry. The discs were washed four times in 20% (w/v) TCA for 30 minutes a time, once with absolute ethanol for 15 minutes and finally with diethylether for another 15 minutes (all washings used about 100ml of washing medium per 100 discs and carried out on ice). The discs were then air dried and counted in Toluene/PPO scintillant (See Section 3.2.3.) using a Packard Tricarb Liquid Scintillation Counter.

3.2.5. Histone Extraction in vivo

Histones were extracted from germinating wheat embryos using the method of Johns (1964) modified as follows:-

Wheat embryos, which had been germinated a known period of time with a known specific activity and amount of ^{3}H-adenosine in germinating medium as described previously (See Section 3.2.1.) were removed and washed twice with germinating medium, dried with filter paper, using an air suction pump, and homogenised in 0.4M H_2SO_4 and extracted in this acid for 1½ hours on ice. The acid extracted material was centrifuged using a Beckman microfuge at 10,000g for 15 minutes. The supernatant was removed and stored on ice. The pellet was re-extracted using 0.4M H_2SO_4 for another 1½ hours on ice, then re-centrifuged as above and both supernatants were combined. Three volumes of 100% enthanol were added to the combined supernatant and stored overnight at -20°C. This was centrifuged as above and the pellet stored at -20°C for four hours to increase the insolubility of the histones. One millilitre of distilled water was added to the pelle and the samples mixed vigorously, then an aliquot of this sample was counted to check the incorporation of isotope into the histones. The resulting sample was used as the soluble total histone preparation.

3.2.6. ADP-Ribosylation of Nuclear Proteins *in vivo*

Embryos were incubated in germination medium as described before (See Section 3.2.1.)with the addition of 20µl of radioactive material (^{3}H-adenosine or ^{3}H-thymidine or ^{3}H-leucine as indicated in the results). Various times were used prior to processing for caesium chloride-urea equilibrium density gradient centrifugation. The embryos were taken out and washed and dried (as described in Section 3.2.5.) and then homogenised in 3mls of 8M urea pH 6.5. The samples were centrifuged at 18,000 rpm for 30 minutes in an MSE 18 centrifuge, then 2.5 mls of the supernatant was mixed with 2.5ml of a solution containing 100% (w/v) CsCl containing 0.2M NaAC/HAC pH 6.5. The solution was thoroughly mixed and left for a further 15 minutes at room temperature, the final concentration being 4M urea and 50% (v/v) CsCl. Equilibrium density gradient centrifugation was carried out at 200,000g for 72 hours at 15°C in either a Beckman L5-65 or L5-50 ultracentrifuge using an SW50.1 rotor. After centrifugation, samples were fractionated from the bottom upwards using a syringe needle carefully lowered to the bottom of the centrifuge tube (Fox & Pardee, 1970) and 10 drops (about 0.5ml) collected at the rate of 12ml/h. Aliquots of these fractions were then precipitated in 20% TCA and counted for radio-activity as previously discussed (See Section 3.2.3.). The

density profile of the gradient was determined by measuring the weight of a 0.1ml sample of each fraction and correcting for its urea content.

3.2.7. Characterisation of Isolated [^{3}H]Adenosine Labelled Material

3.2.7.1. Analysis of Protein Material Isolated from Embryos Incubated with [^{3}H]Adenosine and Centrifuged to Equilibrium in CsCl-Urea

A reaction mixture was made up of 4M urea; 50mM Tris HCl pH 8.2; 20mM glucose-1-phosphate; 20mM $MgCl_2$.

175µl of this solution contained 0.2 I.U. of snake venom phosphodiesterase. At this stage, 25µl of adenosine labelled material (dialysed against 5M urea) from the gradient was also added to the above reaction mixture. This was incubated at 37°C overnight. The snake venom phosphodiesterase is stimulated by the addition of magnesium chloride (Razzell & Khorana, 1959). Glucose-1-phosphate is used as an alternative substrate in large excess, so that monoesterase activity will hydrolyse the glucose-1-phosphate rather than AMP or phosphoribosyl-AMP. The latter are derived from monomer and poly(ADP-ribose).5'-AMP or 5'-GMP are often used as alternative substrate by other workers, however, they tend to inhibit the snake venom phosphodiesterase rather seriously.

3.2.7.2. Alkaline Phosphatase Digestion of Phosphodiesterase Products (To produce nucleoside products from mono nucleotides).

A standard assay of 150µl solution containing the

following was normally used:-
50mM Tris/HCl pH 7.5; 10mM $MgCl_2$; 0.2 International Units of alkaline phosphatase.

This solution was mixed with 50µl of sample to be analysed. The mixture was incubated at 37°C for 4 hours.

3.2.7.3. Base Treatment

175µl of 0.1M NaOH was mixed with 25µl of the appropriate digest and incubated at 37°C overnight.

3.2.8. Aminoethyl Cellulose Chromatography

This system was established to separate monomeric and polymeric material obtained from base treatment of material isolated from adenosine labelled cells (see Section 7.3). Aminoethyl cellulose (an anion-exchange cellulose) was placed in 6M acetic acid and mixed well. This was applied to a 6 x 0.5cm disposable polypropylene column and the column washed well with water. The sample to be analysed was dissolved in 100µl distilled water and applied to the column. Ten millilitres of 6M acetic acid was used to elute AMP and mono ADPR, the column was washed with 5mls of distilled water (to remove acetic acid). Finally 10mls of 1M ethylamine was used to elute poly (ADP-ribose) or other labelled polynucleotide,if this was

present. Five millilitre fractions were collected and lyophilized. They were dissolved in 100µl of distilled water and used for different analytical procedures to characterise the labelled material.

3.2.9. Thin Layer Chromatography

All thin layer separations were carried out on 20cm x 20cm "Baker Flex" PEI-cellulose t.l.c. sheets. Before use, the plates were washed in 2.0M NaCl for 30 minutes, rinsed thoroughly in distilled water and then soaked in distilled water for about an hour. They were finally air-dried. Appropriate standards were run with the experimental sample. The standards were always 5mg/ml made up in 20% (v/v) or 50% (v/v) ethanol to facilitate application onto the plate. Ten microlitre of standard and 10-50µl of sample per track were applied to the origin which was positioned 2cm from the bottom of the sheet. Every time a portion of the sample was applied, the wet band was dried with a hair dryer before any further application of sample was made. The plate was developed in the appropriate solvent and then taken out, dried and the spots visualised under U.V. light (emission 250-280nM). The PEI-cellulose plate was then cut up into 0.5cm sections from the origin to solvent front and placed in scintillation vials. The scintillation fluid was added and the samples

counted as previously described (Section 3.2.3.).

In the case of samples containing high salt, the latter was removed after application of the sample on the plate by soaking the TLC plate in anhydrous methanol for about 30 minutes with subsequent air drying. This procedure was usually carried out after application of the standard (Stone et al., 1973).

3.2.10. Ion-Exchange Thin Layer Chromatography

3.2.10.1. T.L.C. Solvent System

Thin layer chromatography on PEI-cellulose was chosen because it is a well established method for the separation of nucleosides, nucleotides and their derivatives (Randerath & Randerath, 1967). Basically there are many systems to be used, e.g. lithium chloride; borate; acetic acid system and a variety of others (see above reference). The borate system was routinely used in this work to analyse the base digestion as follows.

3.2.10.2. Borate System

The borate/sodiumtetraborate ($H_3BO_3/Na_2B_4O_7$) system of Young & Sweeney (1978) was used to determine the percentage conversion of ADPR to 5'-AMP. This system achieves a good separation of 5'-AMP, 3'-AMP and ADPR (See Figure 3.1.). The latter being an intermediate product

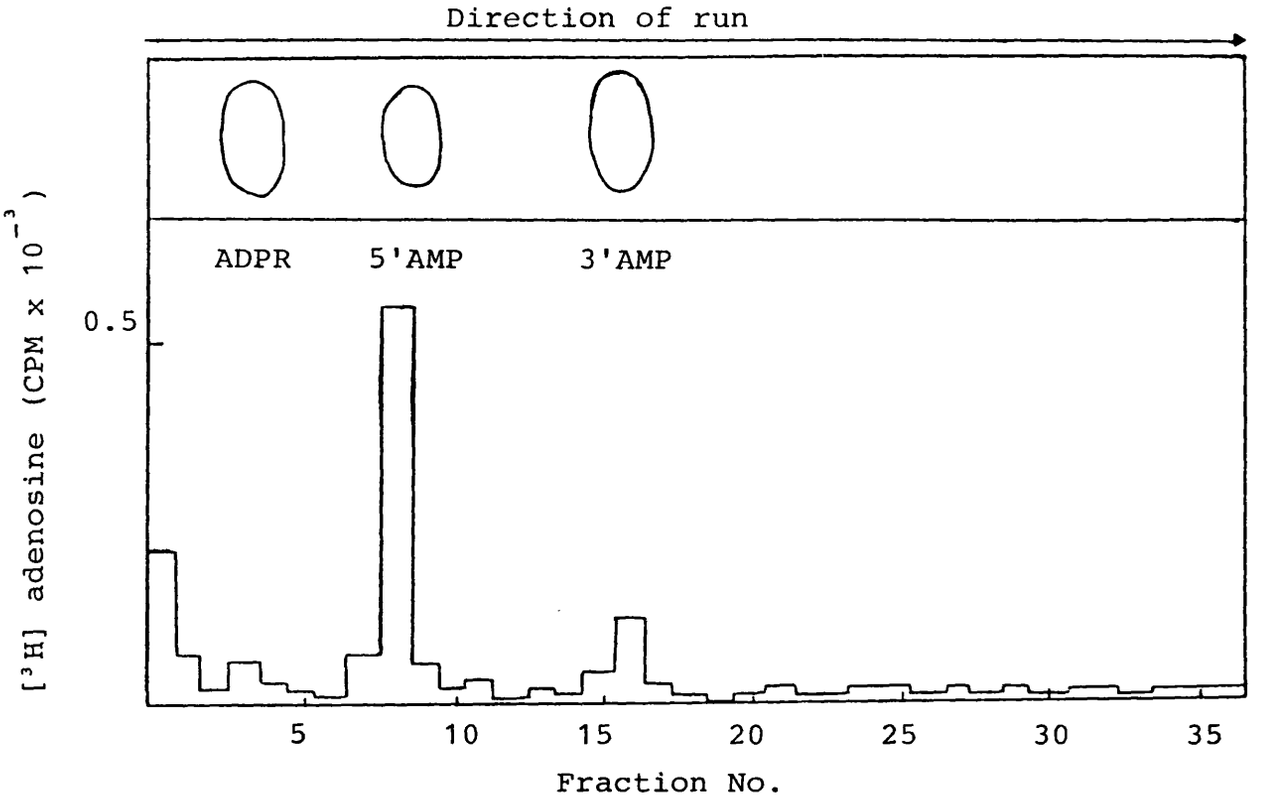


Figure (3.1) 0.I5M sodium tetraborate/0.5M boric acid

T.L.C. of base digest

of base digestion of protein-bound ADPR (Goebel et al., 1977). The separation of 5'-AMP and 3'-AMP is achieved by the fact that 5'-AMP, but not 3'-AMP, is capable of forming a cis-diol complex with borate. This is because 5'-AMP has two free hydroxyl groups at the 2' and 3' positions, whereas 3'-AMP does not (see Figure 3.2). The formation of this complex retards or lowers the mobility of the molecule during T.L.C. After application of the standards and samples, the sheet was placed in a tank containing 50ml 0.1M sodium tetraborate/0.5M boric acid and the solvent front was allowed to run to about 18cm.

3.2.10.3. T.L.C. Counting Procedure

When the plate was dried and the spots visualised under U.V., it was cut into 0.5cm strips. Each strip was placed in a scintillation vial, and 0.5ml of 5% perchloric acid (PCA) was added, and the vials placed in the oven at 70°C for at least 15 minutes. After this heat treatment (which cleaves the N-glycoside bond), 10mls of triton/toluene/PPO scintillant was added and the samples counted for radioactivity (Section 3).

3.2.11. Gel Electrophoresis

3.2.11.1. Gel Scanning

The gels were scanned (using a Pye-Unicam SP 1800 UV

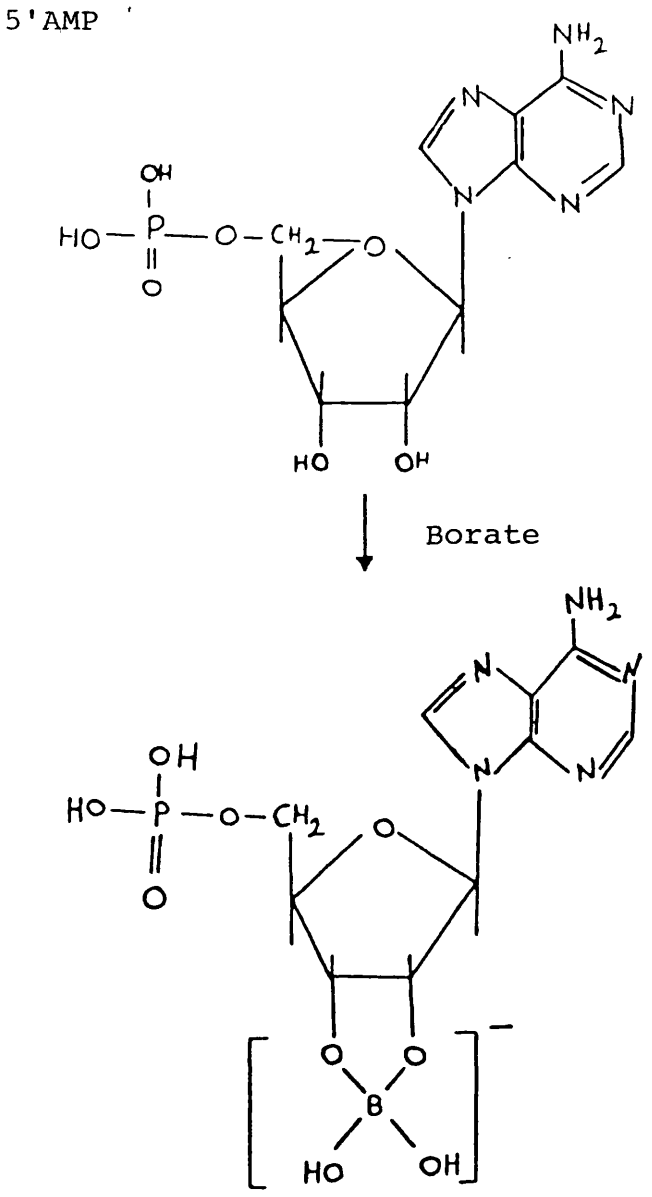


Figure (3.2) Borate complex formation with sugars containing free hydroxyl groups at the 2' and 3' positions.

Spectrophotometer modified to scan gels) at 620nM using a slit width of 1mm and the scans recorded with a Pye-Unicam Linear Recorder.

3.2.11.2. Gel Counting

The gels were frozen on dry ice and sliced into 0.15 cm pieces. Each slice was placed in a scintillation vial and 10ml scintillant was added containing 0.6% (w/v) PPO, 1% (v/v) soluene 350 and 1% (v/v) hyamine hydroxide in toluene. Then vials were tightly capped and incubated for 72 hours at room temperature and finally counted using a Packard scintillation spectrometer. This is essentially the method of Aloyo (1979).

3.2.11.3. Acid-Urea Electrophoresis

The polyacrylamide gels were prepared by mixing 4ml of solution A (60% (w/v) acrylamide, 0.4% (w/v) bis-acrylamide in water) with 2ml of solution B (43.2% (v/v) glacial acetic acid, 4% (v/v) TEMED) and 10 mls of solution C (10M urea, 0.2% (w/v) ammonium persulphate) (Panyim & Chalkley, 1969). The gels were formed in glass tubes of length 10cm and internal diameter 4mm. The gel solution was degassed before pouring and the top of the gel protected from oxygen by a layer of water which also served to keep the gel surface flat. 1.1ml of gel

solution was placed in each tube and allowed to polymerise for about 30 minutes. A 50µl aliquot of histone material was mixed with an equal volume of buffer (30% (w/v) sucrose, 1.8M CH_3CO_2H, 0.1% (w/v) methylene blue) and placed on the top of gel using a Hamilton syringe. The buffer was 0.9M HAC and the upper reservoir was the anode. Electrophoresis was carried out at 2mA per tube at room temperature. Electrophoresis was terminated when the methylene blue marker was almost at the bottom of the tubes. The gels were removed from the glass tubes and left in the stain solution (2.5g Coomassie blue G, 454ml methanol, and 92ml glacial acetic acid made up to 1 litre with water) for at least 8 hours. They were then destained using 0.9M acetic acid at 37°C until the histone bands became clear. The developed gels were then scanned at 620nm and counted.

3.2.11.4. SDS Gel Electrophoresis

This system was used to analyse proteins from the top of the CsCl density gradient. The method employed was a modified system of Weber & Osborn (1969). Gels contained 7.8% (w/v) acrylamide, 0.18% (w/v) methylenebis-acrylamide as cross linker, 0.1% (w/v) SDS, 5M urea and 0.2M sodium phsophate buffer pH 6. The gels contained 10^{-4}mM ammonium persulphate and 0.001% (v/v) TEMED. The gels were dealt with as discussed above. Samples were

dissolved in 10M urea at a protein concentration of 2.6mg/ml. They were incubated at room temperature for about 30 minutes. The solution was diluted with buffer (5M urea and 0.2M sodium phosphate pH 6) to a protein concentration of 1mg/ml, such that the final solution contained 3.8M urea, 7.7mm sodium phosphate buffer pH 6, 0.08% (w/v) SDS, 0.08% (v/v) β-mercaptoethanol, 8% (w/v) glycerol and 0.008% (w/v) bromophenol blue. The resulting solution was incubated for a further 2½ hours at room temperature. The pretreatment with urea alone was necessary to achieve complete solubilisation of protein. The electrode buffer was 0.1M sodium phosphate pH 6 containing 0.1% (w/v) SDS and 5M urea. All solutions containing urea were freshly made up to avoid the formation of cyanate, which changes the conditions of electrophoresis. Samples of 20µl were applied in this system and electrophoresis was carried out at 3mA/gel for the first 10 minutes and then increased to 8mA/gel for the rest of the electrophoresis.

CHAPTER 4

In Vitro Studies on Wheat Embryos

4.1. Introduction

4.1.1. Isolation of Wheat Embryos

Intact wheat embryos were prepared as discussed in Section 3.2.1. The method took approximately 15 minutes to give complete separation of the embryos from other seed fragments. When the embryos were subsequently washed and dried, they had a tendency to stick together, but when they were air-dried, they stayed separate and were easily handled.

This method gave embryos which were always better than 95% viable. They were discarded if their viability fell below 90%. Nuclei were prepared from the viable embryos (See Section 3.2.2).One gram (dry weight) of viable wheat embryos yielded approximately 8×10^7 nuclei when examined using a phase contrast microscope.

4.1.2. Characterisation of the ADPR-Transferase Activity in Isolated Nuclei from Wheat Embryos

The nuclei of wheat embryos catalyse a direct polymerisation of the ADP-ribose moiety of NAD into a polymer, (poly ADP-ribose), with the simultaneous release of nicotinamide. Both the polymer and the enzyme responsible for the polymer production in animal and plants have been reviewed in detail in chapter 1 and 2 of this

thesis.

The work described in this chapter demonstrates that nuclei isolated from wheat embryos possess an ADP-ribose transferase activity which could be affected by some inhibitors, salts and other materials. The enzyme activity was partially characterised with the aim of optimising the production of poly(ADP-ribose) by isolated nuclei in order to facilitate meaningful results in future experiments. The enzyme activity was also looked at, so that some insight into the biological function of protein ADP-ribosylation might be obtained.

The characterisation of the enzyme in wheat embryos nuclei was performed in its entirety before any other experiments were carried out and this formed the basis of a preliminary investigation into the role of ADP-ribose transferase in wheat embryos.

4.1.3. In vitro Studies

Experiments was performed using the standard assay conditions throughout. These were the use of sonicated nuclei (resulting in an increase in activity of the polymerase) in 0.1M triethanolamine (TEA)-HCl, 2mM $MgCl_2$, 10mM βmercaptoethanol, pH 8.2, incubated with 2.5μM [^{3}H] NAD at 26°C.

The nuclei were incubated with [^{3}H]NAD for 20 minutes and then inhibitor was added to the required concentration.

Subsequently, aliquots were removed at intervals and counted for acid insoluble radioactivity as discussed in Section 3.2.4.

Studies on the stability of ADP-ribose transferase and glycohydrolase in frozen nuclei of wheat embryos (see section 4.3) were carried out under almost identical conditions as those for fresh nuclei, the only exception being that before adding the [^{3}H]NAD to the assay, the nuclear preparation was stored at -20°C for differing periods of time.

Many inhibitors have been used with both fresh and frozen nuclei of ungerminated wheat embryos. Germinating wheat embryos were used to try to explain the relationship between DNA metabolism and the effect of the inhibitors on polymerase activity, as well as to study ADP-ribose glycohydrolase activity.

4.2. Results

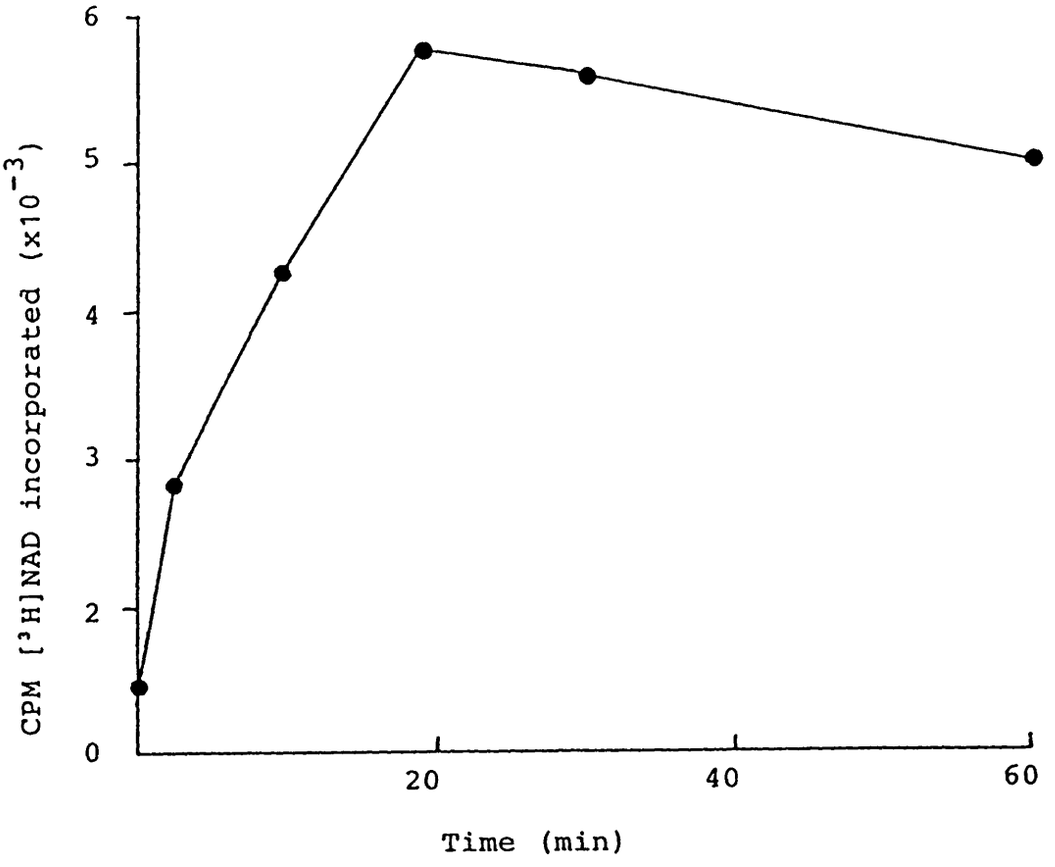
4.2.1. The Activity of ADP-Ribose Transferase in the Nuclei of Wheat Embryos

Figure 4.1. shows a typical time course for ADP-ribose transferase activity when isolated nuclei were incubated with [^{3}H]NAD at 26°C and pH 8.2. This figure indicates that the incorporation of [^{3}H]NAD was almost complete at 20 minutes, after which time there appeared to be a turnover of polymer where the rates of synthesis and

Figure 4.1.

Time course of incubation of a standard preparation of isolated wheat nuclei with [^{3}H]NAD.

Nuclei (1×10^6) from wheat embryos were isolated as described in Section 3.2.2. The incubation was carried out in 0.1M TEA-HCl, 2mM $MgCl_2$, 10mM β-mercaptoethanol, pH 8.2 and the NAD concentration was 20μCi/ml at 20Ci/mmole. Aliquots were removed at the times indicated and acid precipitated (Section 3.2.4.).


6
5
4
3
2
0
CPM [³H]NAD incorporated (x10⁻³)
20
40
60
Time (min)

degradation seemed identical. It also suggests the possibility that both ADP-ribose transferase and the glycohydrolase are inactivated, and that the acid insoluble material is being lost slowly due to base (pH 8.2) hydrolysis of base sensitive ADPR/protein linkages.

4.2.2. Effect of Nicotinamide on ADP-ribose Transferase Activity in Isolated Nuclei

Figure 4.2. shows a time course of ADP-ribose transferase activity where isolated nuclei were incubated with [^{3}H]NAD, using the standard assay conditions (Section 4.1.3.for 20 minutes as in Figure 4.1, then making the preparation to 10mM with respect to nicotinamide. The latter is widely recognised as being an inhibitor of ADP-ribose transferase (Hilz & Stone, 1976). Aliquots were removed during the incubation and counted for acid insoluble radioactivity. The results showed that nuclear preparations from wheat embryos were able to hydrolyse [^{3}H]poly (ADP-ribose) to acid-soluble material. The degradation of poly(ADP-ribose) could be due to poly(ADP-ribose) glycohydrolase activity which would yield ADP-ribose following cleavage of the glycosidic bond. The importance of this graph is that for the first 20 minutes the incorporation of [^{3}H]NAD was linear and then started to decrease. The curve also shows that an initial synthesis of poly(ADP-ribose) occurred, followed by its degradation. When 10mM nicotinamide was added at 20 minutes,

Figure 4.2.

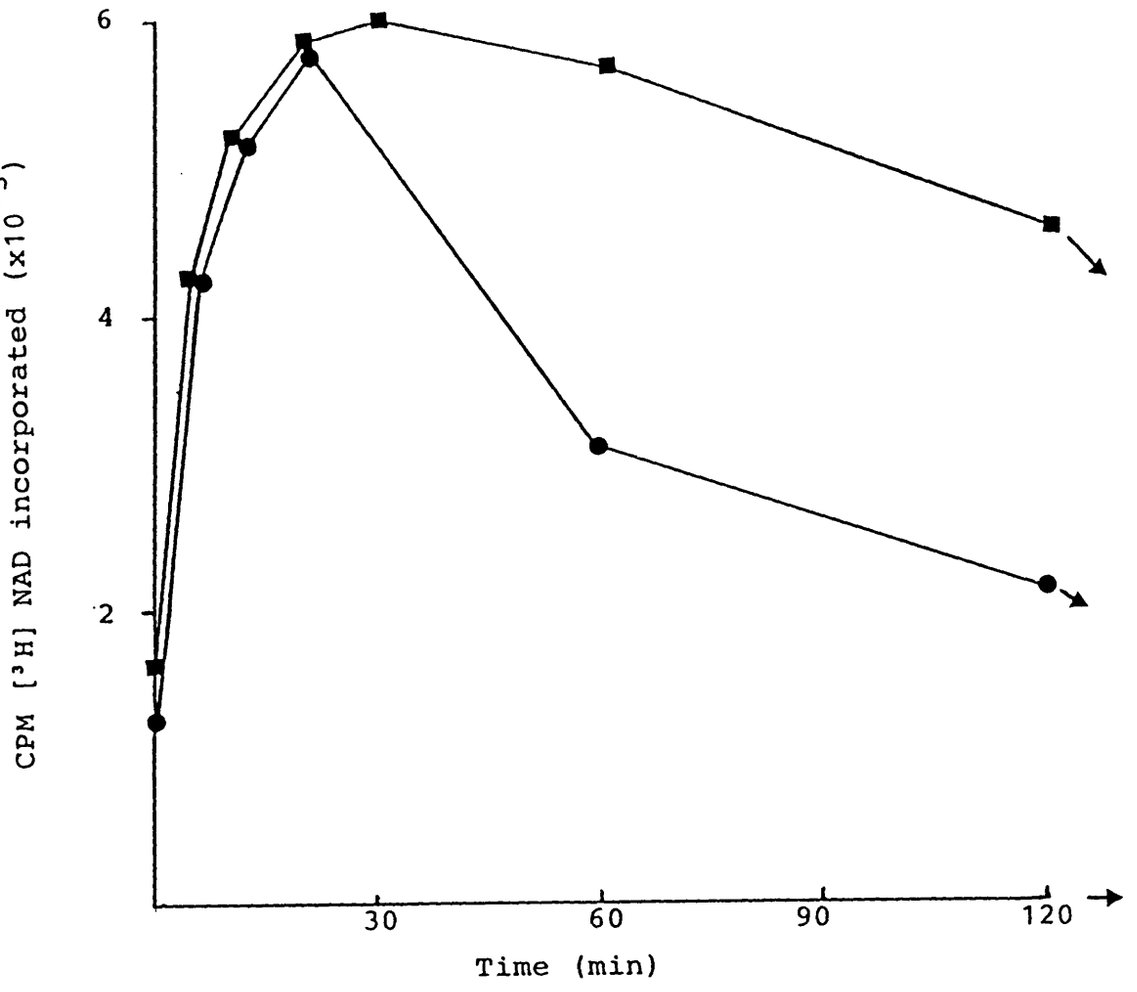
Effect of nicotinamide on poly(ADP-ribose) activity in isolated nuclei.

Isolated nuclei (1×10^6) were incubated in standard assay medium (Section 4.1.3.) with radioactive NAD. Aliquots were removed at the times indicated and acid precipitated. Counts incorporated were determined in toluene/PPO scintillant (Section 3.2.3.).

■——■ control, no additions

●——● nicotinamide added to 10mM at 20 minutes

——▸ represents the counts remaining at 6 hours.


6
4
2
CPM [³H] NAD incorporated (x10⁻³)
30
60
90
120
Time (min)

the rate of decay of acid insoluble material accelerated, indicating that the loss of acid insoluble material may be due in part to poly(ADP-ribose) glycohydrolase. Nicotinamide is however not a good inhibitor of ADP-ribose transferase. At concentrations as high as 100mM some synthesis of poly(ADP-ribose) still occurs (Whitby, 1980). At the time these experiments were being carried out, 3-aminobenzamide was being investigated (in this laboratory) as a potentially specific and powerful inhibitor of ADP-ribose transferase. Figure 4.3 shows that like animal systems, the wheat ADP-ribose transferase is completely inhibited by 3-aminobenzamide at 100μM. Moreover 3-aminobenzamide when added after 20 minutes incubation causes a rapid loss of acid-insoluble material. Under these conditions, the ADP-ribose transferase activity is zero and only poly(ADP-ribose) glycohydrolase is being measured.

It should also be noted that this decay in acid insoluble material proceeds to completion in the presence of 3-aminobenzamide, indicating that the glycohydrolase is removing all the ADP-ribose residues from their protein acceptors. This result is ambiguous since the loss of acid insoluble material may be either enzymic or may be due to base hydrolysis of the ADPR/protein linkage (see also Figure 4.1). This ambiguity is easily resolved as follows. As in Figure 4.2, nuclei were labelled with [^{3}H]NAD for 20 minutes. At this time 3-aminobenzamide and urea (Figure 4.4. curve ▲——▲) were added and the

Figure 4.3.

Effect of 3-aminobenzamide on poly(ADP-ribose) activity in isolated nuclei.

Isolated nuclei (1×10^6) were incubated under standard conditions (Section 4.1.3.) with radioactive NAD. Aliquots were removed at the indicated times and acid precipitated. These samples were counted as described in Section 3.2.3.

■——■ control nuclei, no additions

△——△ 3-AB added to a final concentration of 100μM at 20 minutes.

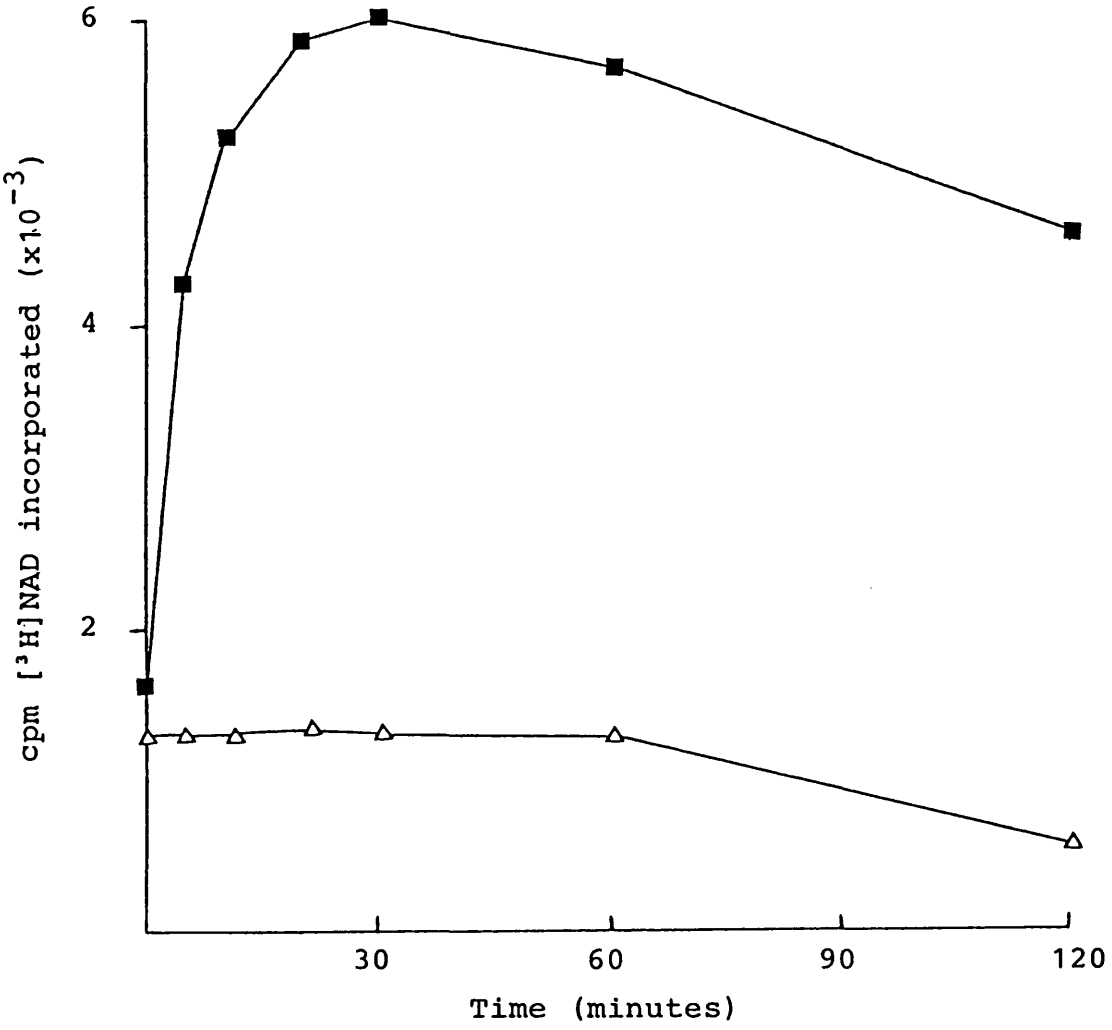
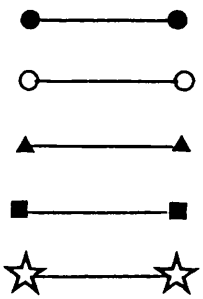

6
4
2
cpm [³H]NAD incorporated (x10⁻³)
30
60
90
120
Time (minutes)

Figure 4.4.

Effect of 3-aminobenzamide on poly(ADP-ribose activity in isolated wheat nuclei

Nuclei (1×10^6) were incubated under standard conditions (Section 4.1.3. and Figure 4.3. legend). After twenty minutes incubation, various inhibitors were added. Aliquots of the resulting incubations were removed and acid precipitated at the times shown on the graph. The radioactivity remaining in the samples was determined as described in Section 3.2.4.



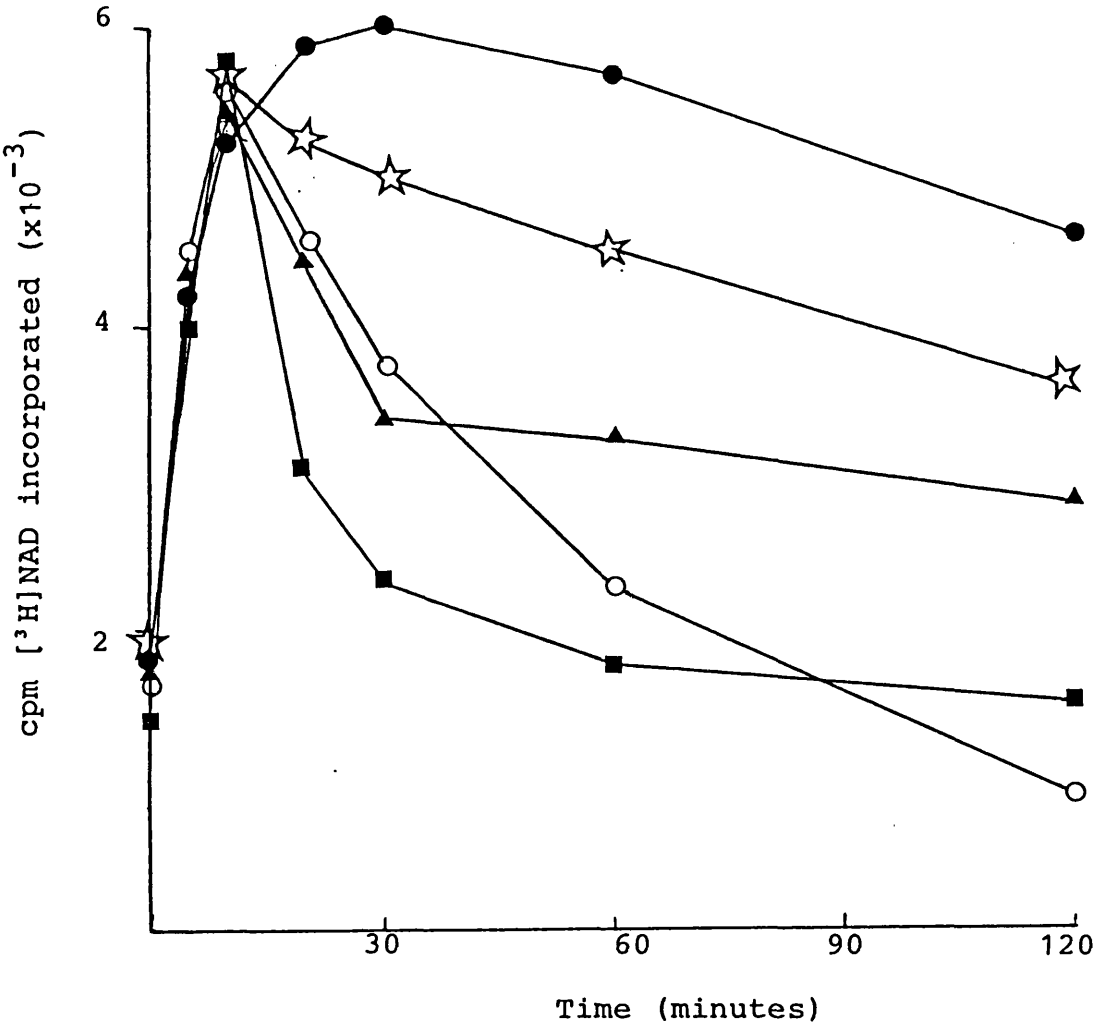
control, no additions

3-AB added to 100μM at 20 minutes

3-AB added to 100μM plus urea to 8M

3-AB added to 100μM plus NaCl to 3M

3-AB added to 100μM plus NaCl to 1M and acetic acid to pH 4.


6
4
2
cpm [³H]NAD incorporated (x10⁻³)
30
60
90
120
Time (minutes)

time course continued. The 3-aminobenzamide inhibits ADP-ribose transferase and urea at 8M will probably completely inhibit both the glycohydrolase and the polymerase. Thus decay in acid-insoluble material which occurs is due only to chemical hydrolysis of the ADPR/ protein linkage. The overall loss of acid-insoluble material here is half (3700 cpm per time) that observed with 3-aminobenzamide alone. As a further check, the pH of the incubation medium was reduced after 20 minutes of [^{3}H]NAD incorporation. When sufficient acetic acid was added at 20 minutes to reduce the pH to 6.0, it was found that the decay of acid insoluble material was really rather slow (☆—☆). These results confirm the fact that under the normal incubation conditions of the ADP-ribose transferase assay, the loss of acid insoluble material is due to both enzymic (glycohydrolase and perhaps diesterase and protease) and chemical hydrolysis. Even under acidic conditions, pH 6 (Figure 4.4 curve ☆—☆), the rate of loss of acid insoluble material is still appreciable. To gain further insight into events which seem to be occuring in the incubation mixture, an experiment was performed where high salt (3M NaCl) was used in an attempt to inhibit the loss of acid insoluble material.

As can be seen from Figure 4.4. curve ■—■ the high salt causes an unexpectedly rapid decay in acid insoluble material which is far quicker than with urea, or

with NaCl.HAC. With these data, it is only possible to conclude that the high salt dissociates ADPR/protein from the nuclear complex thus making it more accessible to the poly(ADP-ribose) glycohydrolase. Of course, the assumption here is that the rate of base hydrolysis is unchanged by the addition of NaCl, which is a valid assumption. Further information about the glycohydrolase activity might have been gained by using two effective (in animal systems, Purnell, 1981) inhibitors of this enzyme. Cyclic AMP at 25μM can be seen to have qualitatively quite a significant effect on the decay of acid-insoluble material (Figure 4.5 curve ▲——▲). The experimental design was the same as before. The cyclic AMP appears to reduce the decay of acid-insoluble material. The significance of this observation is enhanced when the experiment is repeated with ATP. ATP is a relatively poor inhibitor of glycohydrolase and this is certainly the case when one compares curve ■——■ (ATP) with curve ▲——▲ (cyclic AMP) in Figure 4.5.

The experiments outlined in Figures 4.4 and 4.5 confirm that the decay of acid-insoluble material seen when the ADP-ribose transferase is inhibited is due to both enzymic and base hydrolysis. The data with such experiments, of course, must be viewed with some caution. The system used is a crude one (that is, isolated nuclei prelabelled with [^{3}H]NAD) which possibly could be improved if pure polymer was used together with a purified

Figure 4.5

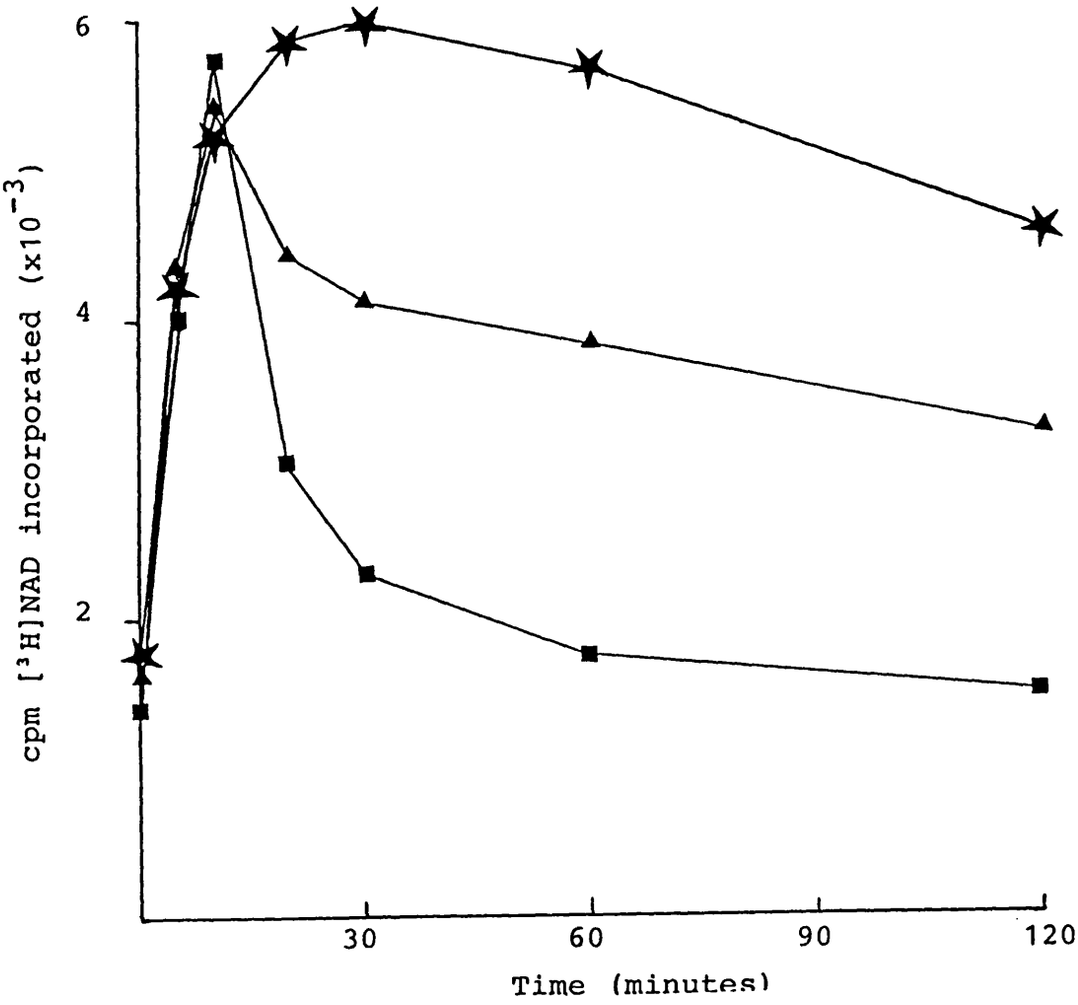
Effect of 3-aminobenzamide with ATP or cyclic AMP on poly(ADP-ribose) activity in isolated wheat nuclei

Nuclei (1×10^6) were incubated under standard conditions with radioactive NAD (Section 4.1.3. and legend to Figure 4.1.). After 20 minutes incubation, various inhibitors were added and incubation continued. Samples were removed at the times indicated, acid precipitated and counted as in Section 3.2.4.

★——★ control, no additions

▲——▲ 3-AB added to 100μM and 3'-5'cAMP made to 25μM final concentration.

■——■ 3-AB added to 100μM and 5'AMP added to a final concentration of 100μM.


6
4
2
cpm [3H]NAD incorporated (x10-3)
30
60
90
120
Time (minutes)

preparation of glycohydrolase. The problem then is that "the system" is even further removed from the in vivo situation than isolated nuclei. An added problem is that a detailed kinetic examination of the glycohydrolase is not possible because of the fact that isolated nuclei contain unknown quantities of endogenous cold polymer (Pearson's group, 1978) i.e. when nuclei are incubated with [^{3}H]NAD, the hot polymer will add to new initiation sites as well as extending pre-existing cold ADPR/protein. Thus whether exogenous hot polymer is used or the semi-endogenous system described here is used, meaningful quantitative analysis of the glycohydrolase cannot be carried out because the exact amounts and chain lengths of polymer are unknown. A further important problem is that the glycohydrolase in animal systems does not remove the final ADP-ribose residue next to the protein (Purnell et al., 1980). In wheat embryos it is not known if this last residue is removed by glycohydrolase or not. It would appear from the various curves presented so far (Figures 4.2, 4.4, 4.5) that the final ADP-ribose residue is, in fact, removed by the glycohydrolase since at 120 minutes, there is no acid insoluble material left (e.g. Figure 4.5 curve ■———■). However it must be appreciated that this system is complicated by the relatively high level of base hydrolysis of ADPR/protein which occurs.

4.3. Inactivation of Glycohydrolase

The isolation of nuclei from wheat embryos (Section 3.2.2)is tedious and somewhat variable with regard to the numbers of nuclei obtained and their quality. In an effort to alleviate this problem, it was thought that nuclei could be isolated in large quantities and then frozen for future use. When this was done, it was found that the incorporation curve was not the typical "bell-shaped curve" but rather one where the activity first increased and then plateaued, remaining constant for at least 6 hours (Figure 4.6). Once again there are technical problems involved in using frozen nuclei, the largest of these being that if nuclei are frozen and thawed, one can show, using phase contrast microscopy, that the nuclei completely break up. Therefore quantitative kinetic comparisons between frozen and fresh nuclear preparations are simply not possible.

The frozen nuclear preparation was further studied as follows. It was found that ADP-ribose transferase activity was stable to freezing whereas the poly(ADP-ribose) glycohydrolase was almost competely inactivated by freezing (Figure 4.7). This is a useful finding for further studies involving the ADP-ribose transferase. However an unexplained and somewhat surprising observation is that a frozen nuclear preparation produces slightly less acid-insoluble material than the corresponding

Figure 4.6

Time course for a preparation of frozen isolated nuclei incubated with [^{3}H]NAD.

Nuclei were isolated and then frozen (Section 3.2.2.). The equivalent of 1 x 10^6 of such nuclei were then assayed under standard conditions (Section 4.1.3.). Aliquots were removed at the times indicated on the graph, acid precipitated and counted as described in Section 3.2.4. The arrow indicates the acid-insoluble counts which remain after six hours incubation.

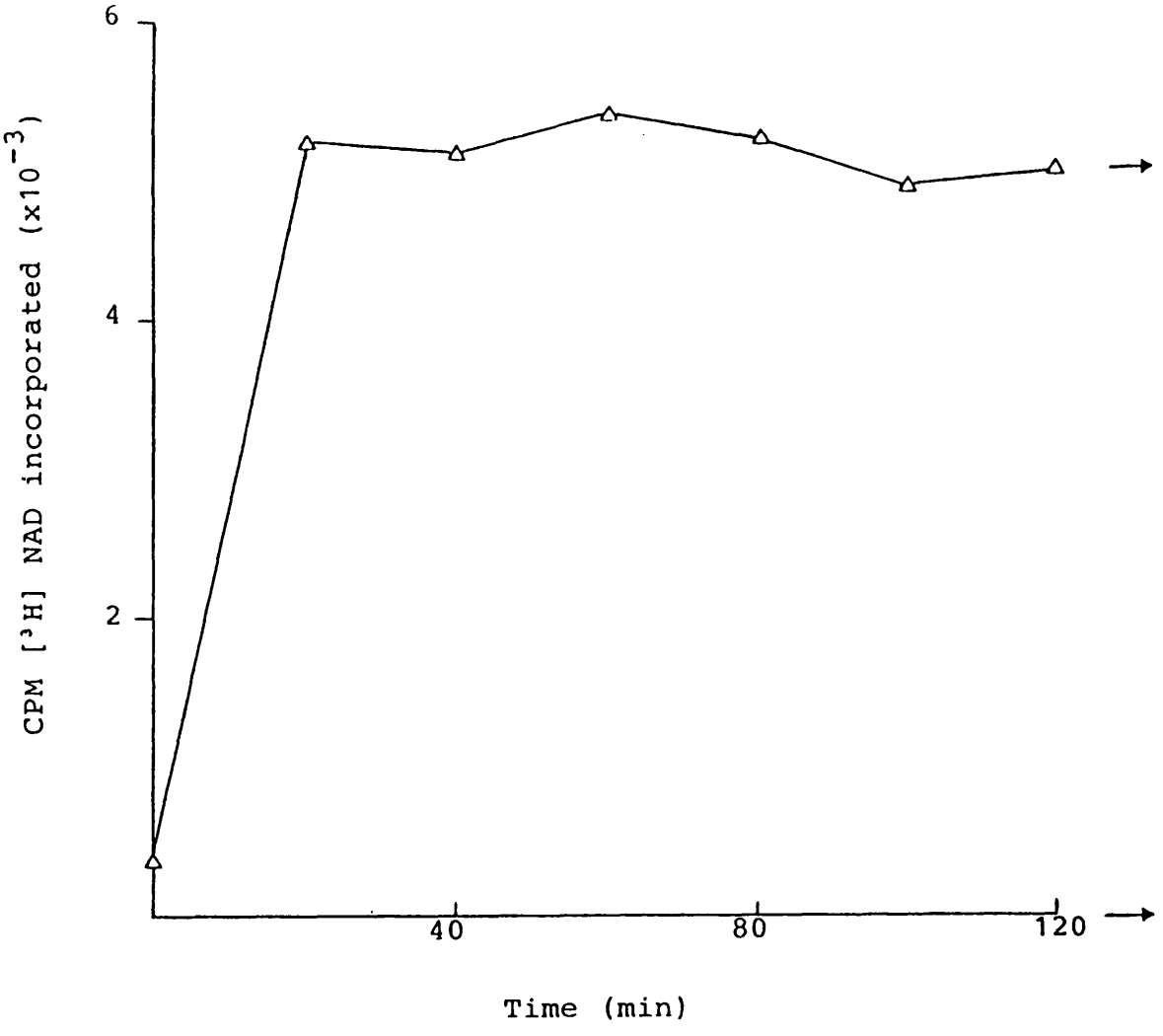

6
4
2
CPM [³H] NAD incorporated (x10⁻³)
40
80
120
Time (min)

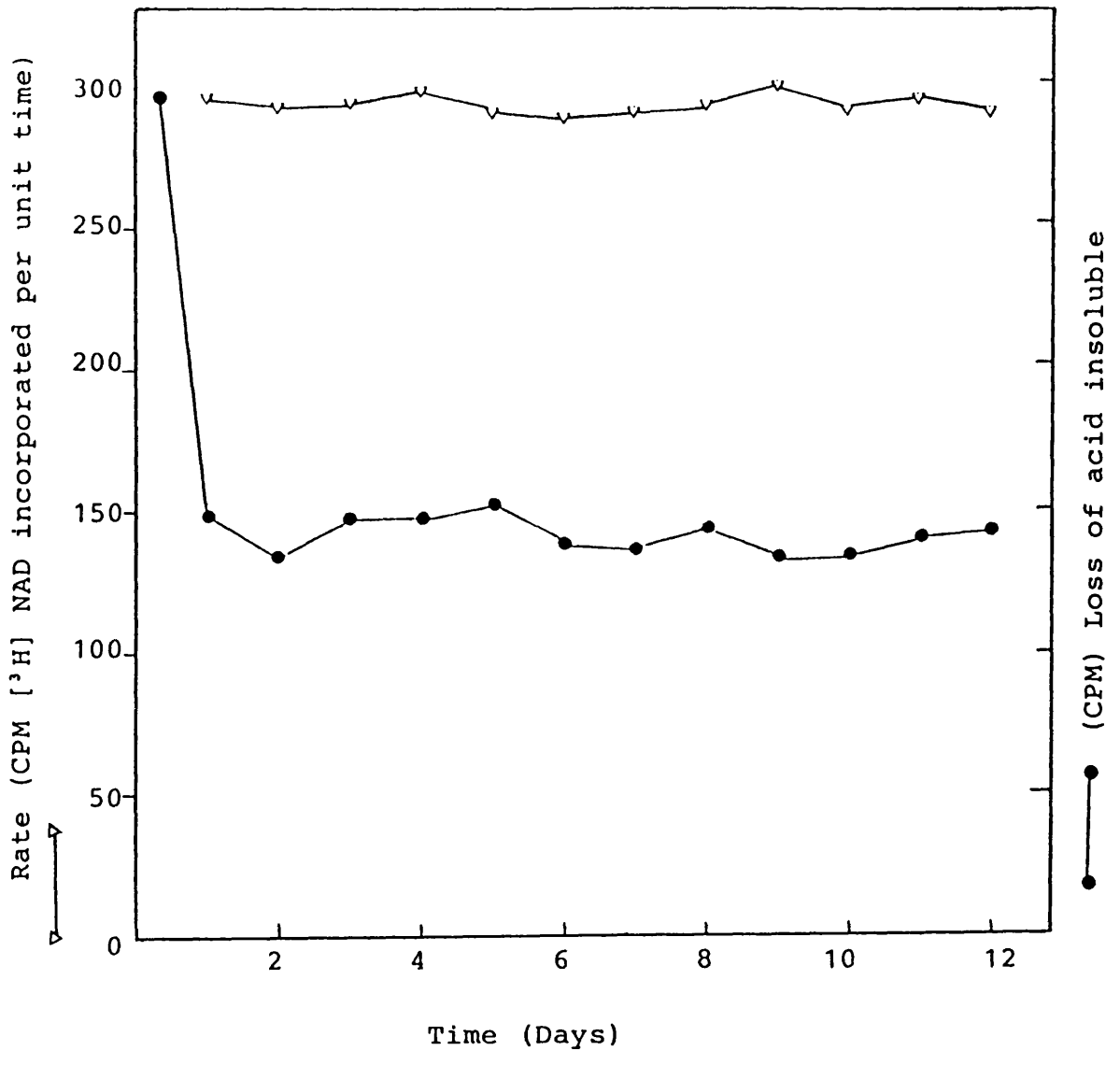
Figure 4.7.

Stability of ADP-ribose transferase and glycohydrolase at -20°C

Nuclei were isolated (Section 3.2.2.) and frozen at -20°C (Section 4.3.). At the times shown on the graph, the nuclei were thawed and incubated with radioactive NAD under the standard conditions. (Normally 1 x 10^5 nuclei was used at each time). After 5 minutes incubation (a time which gave linear rates of radiolabelling), the whole incubation mixture was acid precipitated and counted (Section 3.2.4.).

▽——▽ The rate of incorporation of [^{3}H]NAD into acid insoluble material. A measure of ADP-ribose transferase.

●——● After 5 minutes of the standard incubation conditions, 3-AB was added to 100μM. Aliquots were then removed at 10 minute intervals over a period of an hour. The glycerhydrolase activity is expressed as a loss in CPM from the acid-insoluble material at 20 minutes (100% level).


Rate (CPM [³H] NAD incorporated per unit time)
300
250
200
150
100
50
0
2
4
6
8
10
12
Time (Days)
(CPM) Loss of acid insoluble

equilvalent amount of fresh nuclear preparation. One would expect a large amount of polymer since there is the same amount of ADP-ribose transferase and no ADP-ribose glycohydrolase. One explanation of this finding is that, in nuclei, the limiting substrates are the acceptor proteins i.e. synthesis of acid-insoluble material must stop when the available acceptor proteins have been totally depleted. (See also Figure 4.8.).

The studies outlined in this Section illustrate the difficulty in doing meaningful enzymological studies on isolated nuclei. However such studies are the only possibility at present because more meaningful work using permeabilised cells is quite impossible with plant systems. It must be kept in mind that isolated nuclei are not a good system to work with because:-

(a) the isolation procedure breaks the DNA and activates the polymerase (Berger's group, 1978);

(b) the whole cell compartmentalisation is lost and many nuclear control proteins may be removed (see, [d]);

(c) the substrate(s) concentrations used *in vitro* cannot be the same as those used by the cell *in vivo* i.e. isolated nuclei used [^{3}H]NAD alone whereas even in permeabilised cells other molecules related to NAD (nicotinamide, NADH etc.) are present, even though they are diluted relative to normal cells;

(d) the acceptor proteins for ADP-ribose have not been

Figure 4.8.

Effect of 3-aminobenzamide on poly(ADP-ribose) activity in frozen isolated wheat nuclei

Nuclei were isolated (Section 3.2.2.) and frozen at -20°C (Section 4.3.). The nuclei (1×10^5) were thawed and incubated with radioactive NAD under the standard conditions (Section 4.1.3. and Figure 4.3. legend). After twenty minutes incubation, various inhibitors were added. Aliquots of the resulting incubations were removed and acid precipitated at the times shown on the graph. The radioactivity remaining in the samples was determined as described in Section 3.2.4.

●——● control, no additions

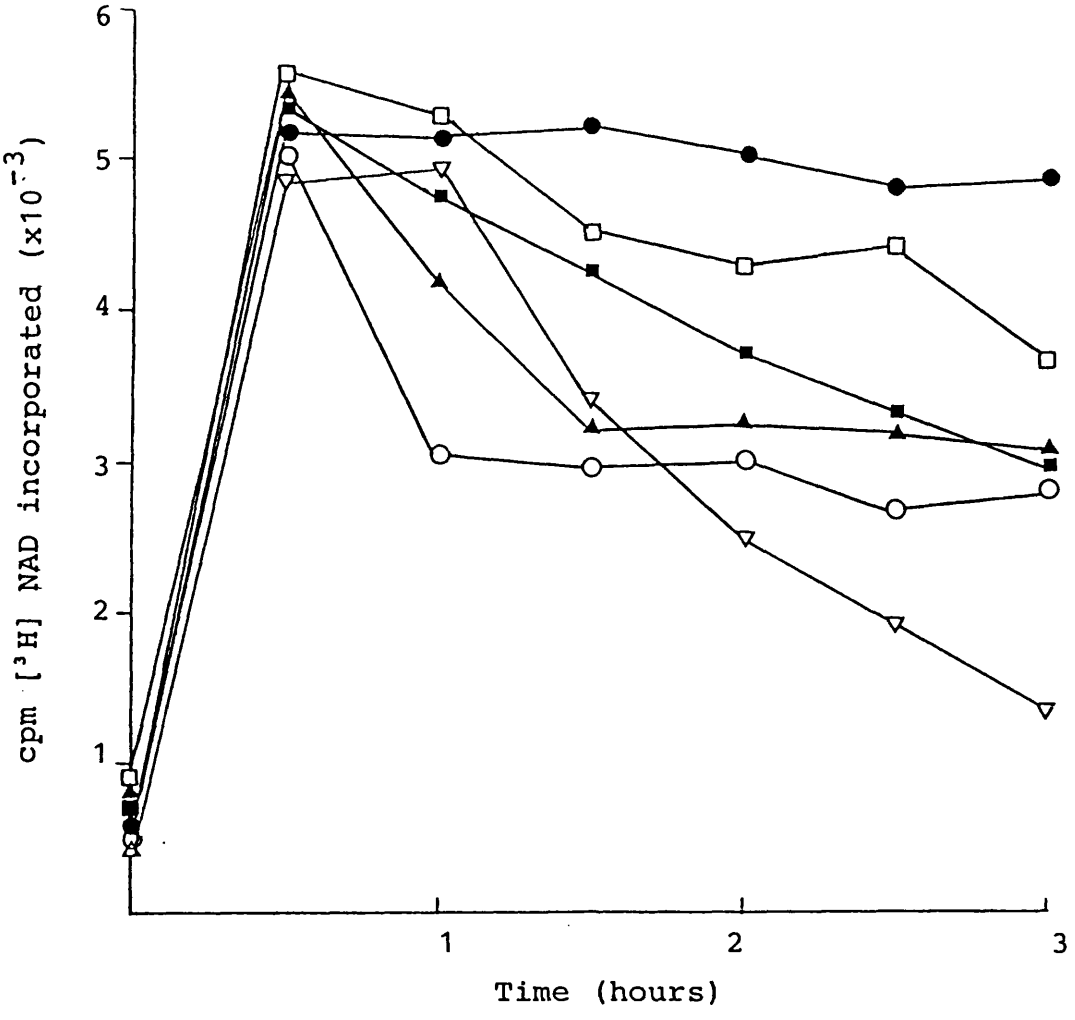
○——○ 3-AB added to 100μM at 20 minutes

▲——▲ 3-AB added to 100μM plus urea to 8M

▽——▽ 3-AB added to 100μM plus NaCl to 3M

■——■ 3-AB added to 100μM plus NaCl to 1M and acetic acid to pH 4.

□——□ 3-AB added to 100μM plus NaCl to 1M and hydroxylamine to 2M.


6
5
4
3
2
1
cpm [³H] NAD incorporated (x10⁻³)
1
2
3
Time (hours)

characterised in any detail (except the histones, (Purnell *et al.*, 1980)). Many of these may be removed during the nuclear isolation procedure;

(e) because the cell compartmentalisation is destroyed when the nucleus is isolated, proteins which do not normally act as ADP-ribose acceptor proteins may in fact act as such *in vitro*;

(f) other enzymes (phosphodiesterase and proteases) which may not have access to ADPR/protein *in vivo* will certainly have access and hydrolyse these ADPR/protein complexes *in vitro* in isolated nuclei.

All the effects outlined above make the use of isolated nuclei for detailed quantitative studies somewhat suspect (Purnell *et al.*, 1980).

It is for these reasons that a study of ADP-ribosylation was attempted using viable wheat embryos as a system *in vivo*.

CHAPTER 5

In Vivo Studies

5.1 Introduction

Isolated wheat embryos germinate normally and behave like whole seeds (Chen & Osborne, 1970). The major problem with whole seed studies is that the starch and outer seed layers are only slowly permeable to water. Thus germination times in whole seeds are delayed by some hours with respect to germination times in isolated embryos. The whole seed also appears to be partially permeable (or very slowly permeable) to precursors like thymidine, leucine, uridine and adenosine. This makes labelling experiments particularly difficult (Figure 5.2). On the other hand the embryo is relatively easily removed from the whole seed (see Material and Method Section3.2.1). Much work has been done to show that embryos also develop normally (Whitby, 1980).

The criteria used in this work for successful germination are that on imbibition of water, root hairs appear at about 20 hours and that the plumules swell prior to shoot formation (see diagram 1).

In plant biochemistry terms, the seed embryo can be likened to the permeabilised animal cell. The wheat embryo is more permeable than the seed and is still viable. Here the analogy ends, because the wheat embryo is not of course permeable to nucleotides in the same way as the

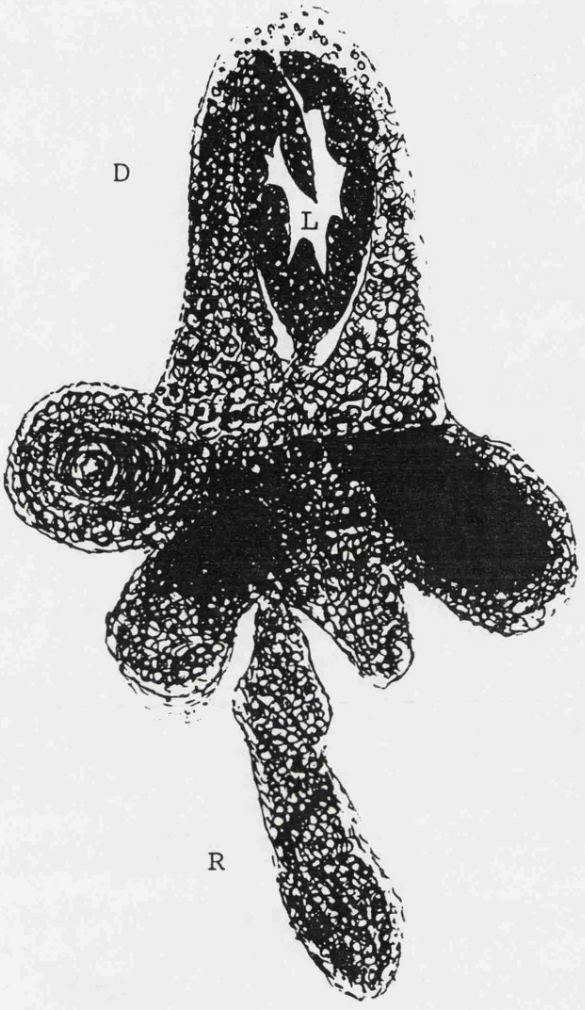


Diagram 1 Germinated wheat embryo: a cross section
(24 hour germination)

Key: D: dividing region
L: leaf
R: root

permeabilised cell is. Thus, work in vivo is restricted to those precursors and inhibitors which will cross the plant cell wall and membrane.

5.2. Germination Studies

Experiments will now be described which attempt to show and characterise ADP-ribosylation in vivo. Figure 5.1 shows the time course of germination using the above criteria. Thus, in 24 hours, over 95% germination occurs.

5.3. Germination and ADP-Ribosylation in vivo

Two main approaches were taken to study ADP-ribosylation in vivo in this work. One involves the use of inhibitors of ADP-ribose transferase as probes and the other approach uses radioisotopic precursors in an attempt to find and characterise ADPR/protein complexes in the wheat embryo.

5.3.1. Inhibition Studies in vivo

Since the present aims of this work are to study the relationship of protein-ADP-ribosylation and cellular function, the first experiments were designed to see if there was any detectable relationship at all between ADP-ribose transferase activity and germination. In these experiments wheat embryos were germinated in a medium which contained known inhibitors of ADP-ribose transferase.

Figure 5.1

Time course for germination of wheat embryos

100 embryos were placed on a petri dish containing germination medium (Section 3.2.1.). An embryo was judged as having successfully germinated when root hairs and plumules appeared (Section 5.1.). At each time shown on the graph (—▼—) the successfully germinated embryos were counted and plotted as a percentage of the total embryos on the petri dish (always 100 embryos). The germination was always carried out in the dark. The curve is representative of 90 germinations.

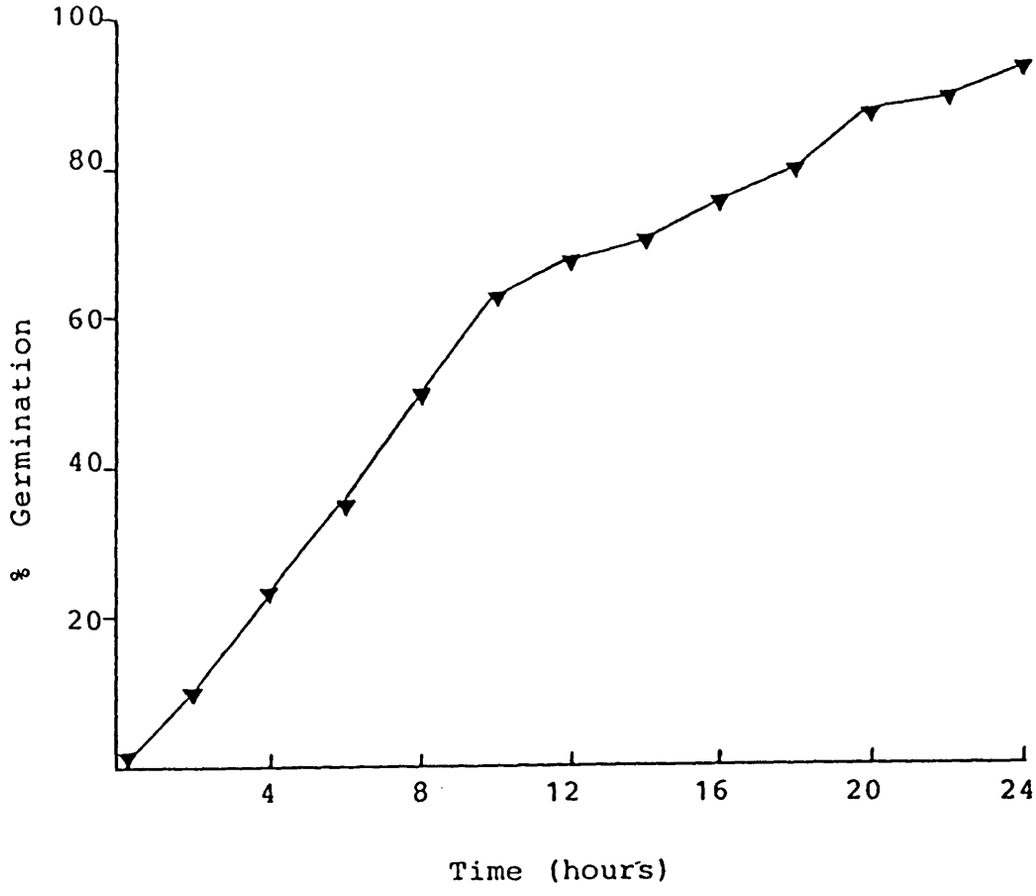
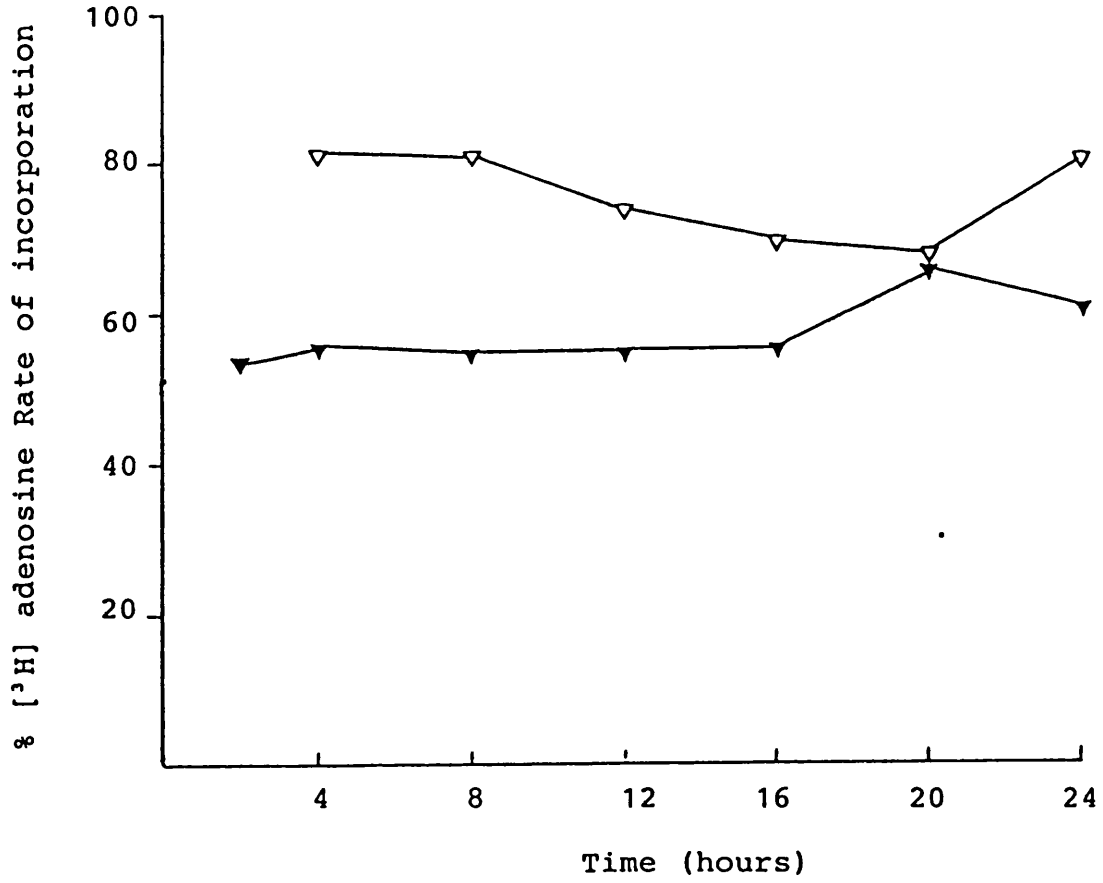

100
80
60
40
20
% Germination
4
8
12
16
20
24
Time (hours)

Figure 5.2.

The effect of thymidine on ^{3}H-adenosine incorporation into DNA (▽——▽) and DNA + RNA (▼——▼).

One hundred embryos were placed in a petri dish containing 5 mls of germination medium and 4mCi/ml [2-^{3}H] adenosine (22Ci/mmole). To one such petri dish was added thymidine to a final concentration of 10mM (test). The control dish contained no thymidine. At various times (see graph 5.1.), embryos were taken and homogenised in a Potter homogeniser in the presence of 20% TCA. The radioactivity incorporated into acid insoluble CPM was determined on this homogenate as outlined in Section 3.2.4. At each time point, the control was always the 100% value and the test (with thymidine) expressed as a percentage of the control value. To determine DNA alone (▽——▽) the embryos were homogenised in 0.1M NaOH, left at 37°C overnight and then acid precipitated. The control embryos were taken at 100% at each time point.


% [³H] adenosine Rate of incorporation
100
80
60
40
20
4
8
12
16
20
24
Time (hours)

Germination was therefore attempted in a medium containing 10mM nicotinamide (Figure 5.3), 5mM 3-aminobenzamide (Figure 5.4) and 10mM thymidine (Figure 5.5).

As can be seen from these Figures (5.3, 5.4, and 5.5), none of these inhibitors affect the rate of germination nor the percentage viability of the embryos. The first conclusion must be that the inhibitors were not entering the embryos. To test this, embryos were grown in ^{14}C-nicotinamide in one experiment and in [^{3}H] thymidine in another. As can be seen from Figures 5.6 and 5.7, these two molecules enter the embryos. The nicotinamide will eventually be incorporated into all the pyridine nucleotides (not analysed in this work) whereas the thymidine enters DNA. The latter was easily tested by acid precipitating embryos which had been incubated in [^{3}H]-thymidine (see later, Section 5.3.2.).
The 3-aminobenzamide was not available to radiolabel, so a less sensitive detection method had to be used. Here embryos were incubated in 2mM 3-aminobenzamide and at various times, 20 embryos were removed, washed in cold germination medium until all extra-embryo 3-aminobenzamide had been removed. The embryos were then homogenised in absolute alcohol in a tight fitting glass-glass potter homogeniser. The homogenate was centrifuged in a bench centrifuge at 1000g for 10 minutes. The alcohol supernatant was spotted onto Whatman No.1 filter paper and observed under UV light. 3-Aminobenzamide is intensely

Figure 5.4.

Time course for germination of wheat embryos, with/without 3-aminobenzamide

Embryos were germinated under standard conditions (Section 3.2.1. and Figure 5.1.). No additions were made to the control germination (O——O), while the test germination contained 5mM 3-AB (▽——▽).

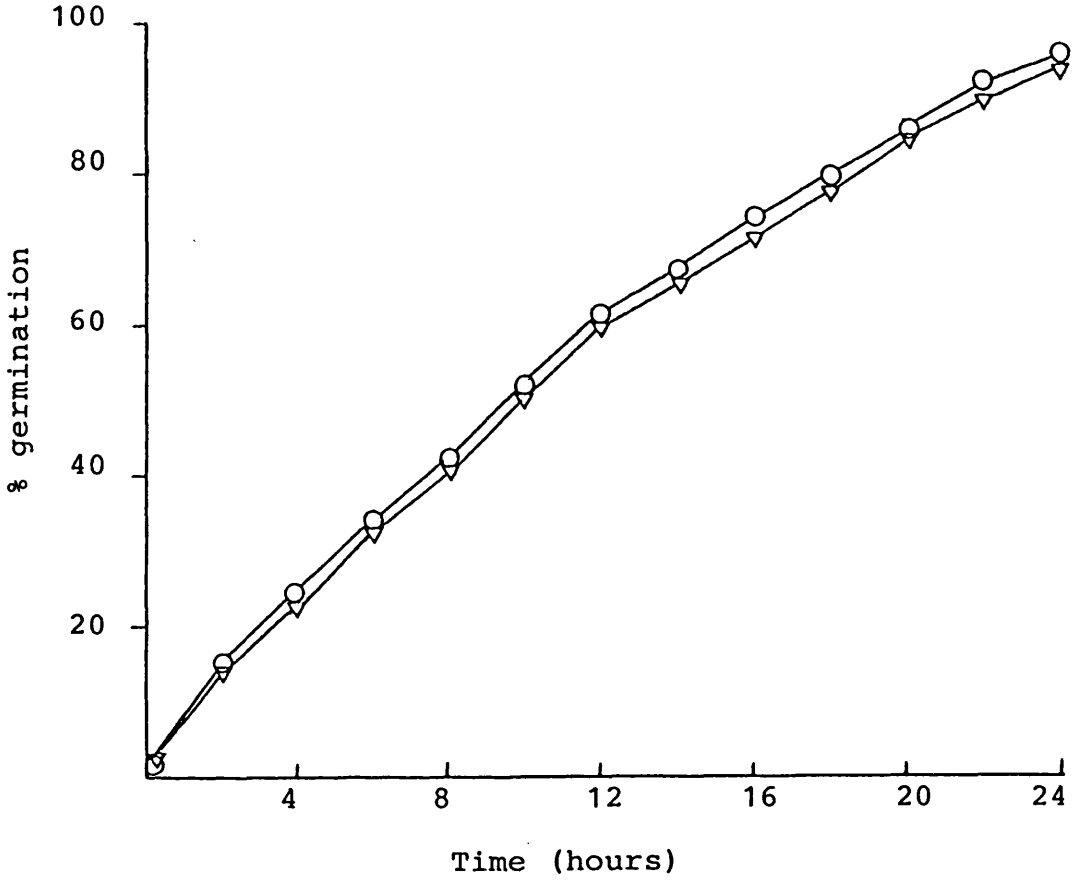

100
80
60
40
20
% germination
4
8
12
16
20
24
Time (hours)

Figure 5.5.

The effect of 10mM thymidine on germination of wheat embryos

Embryos (100) were germinated in petri dishes under standard conditions (Section 3.2.1. and Figure 5.1.). The control germination had no additions (■——■). The test germination contained thymidine at a concentration of 10mM (□——□).

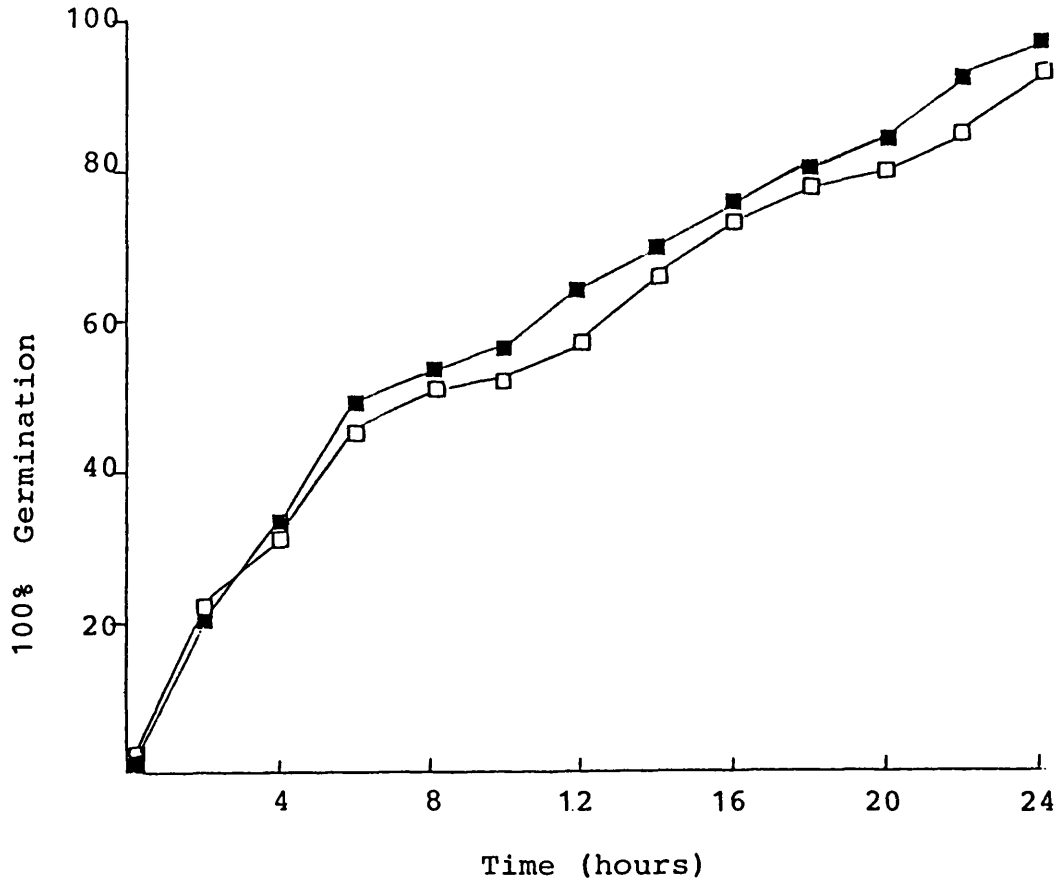

100
80
60
40
20
100% Germination
4
8
12
16
20
24
Time (hours)

Figure 5.6.

The effect of ^{14}C-nicotinamide on the germination of wheat embryos

Embryos (100) were germinated under standard conditions (Section 3.2.1. and Figure 5.1.). The control germination contained no additions (●——●). The test germination contained 5mM [^{14}C]nicotinamide (1μCi/μmole and 5μCi/ml). At the times indicated (—□—), 2 embryos were removed, washed and then homogenised in 2 mls germination medium. Aliquots of 100μl were added to 2ml triton/toluene/PPO (Section 3.2.4.) and counted directly.

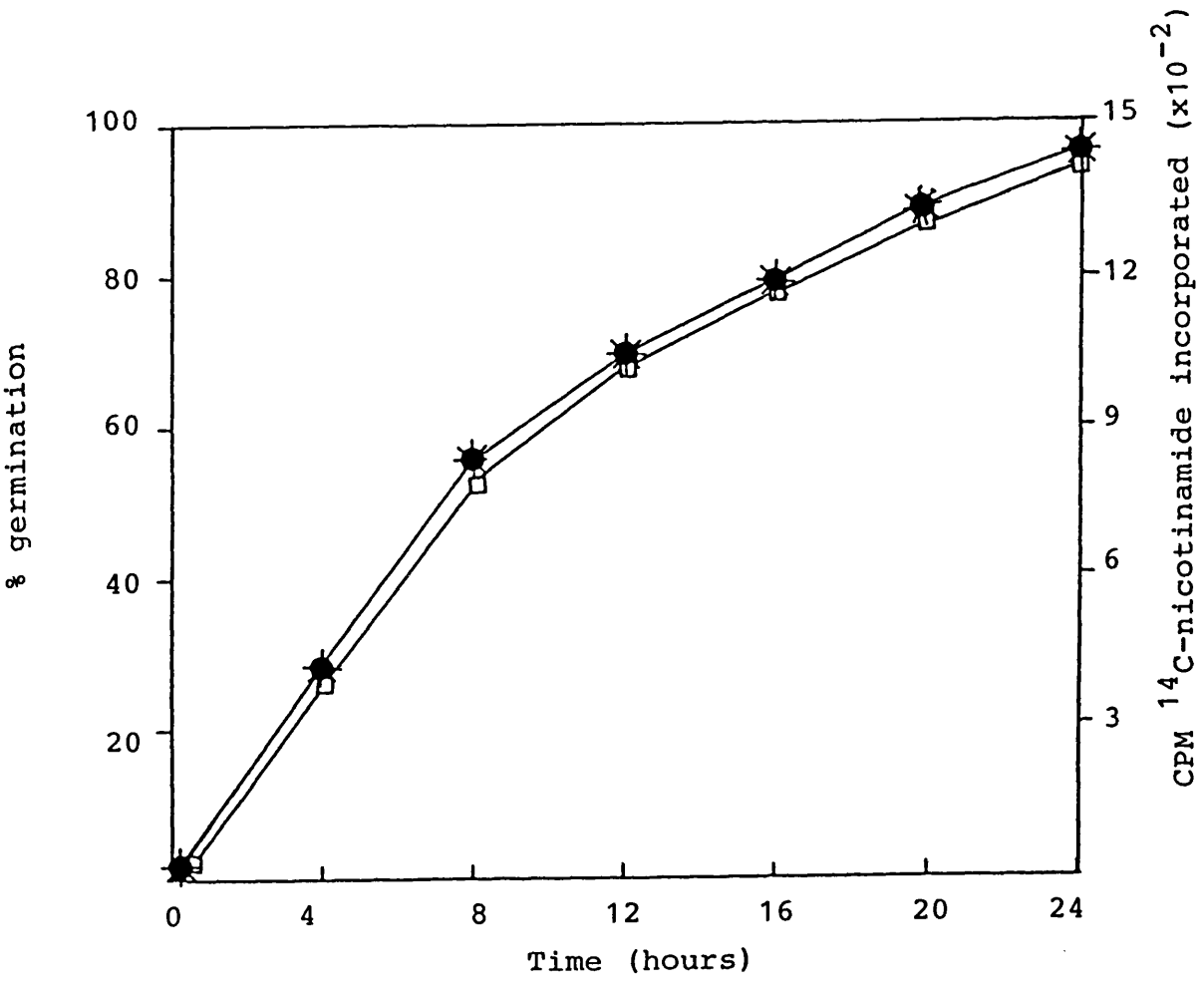
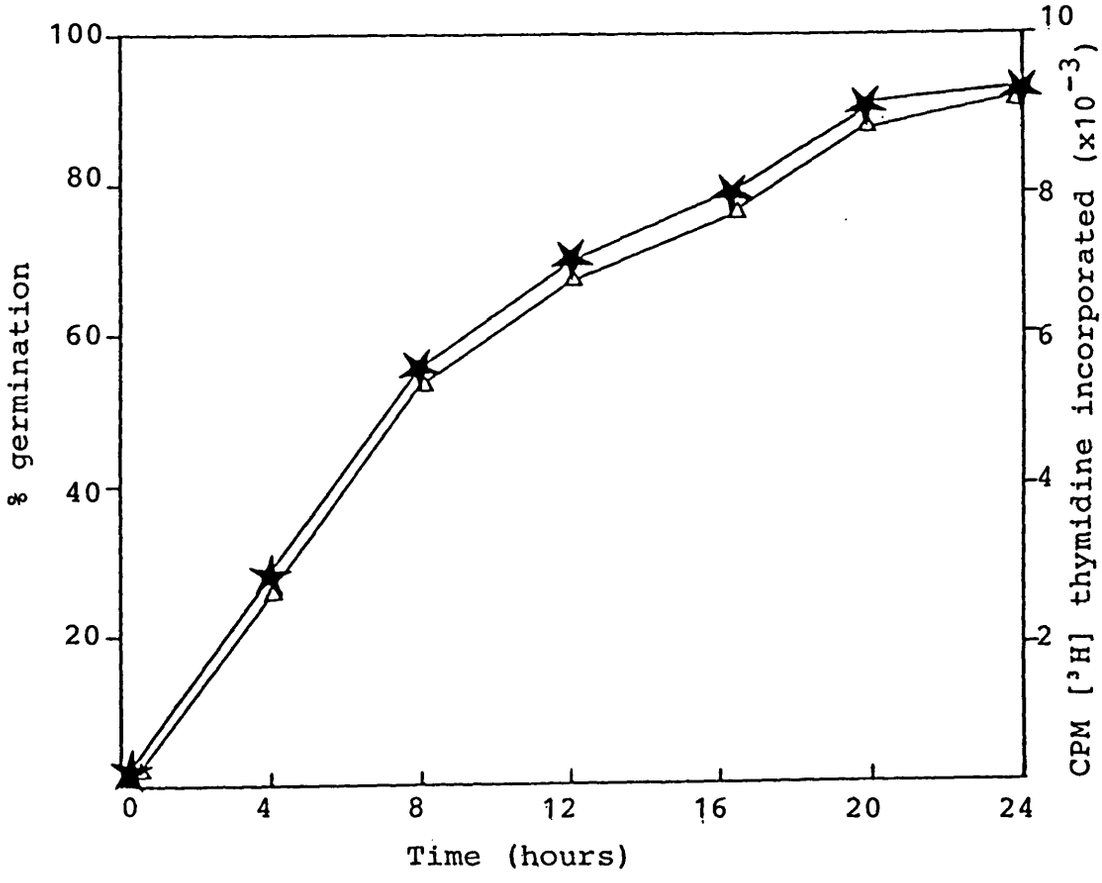

% germination
100
80
60
40
20
0
4
8
12
16
20
24
Time (hours)
15
12
9
6
3
CPM ^{14}C-nicotinamide incorporated (x10^{-2})

Figure 5.7.

The effect of [^{3}H]thymidine on the germination of wheat embryos

Embryos (100) were germinated under standard conditions (Section 3.2.1. and Figure 5.1.). The experimental system was identical to the previous experiment (Figure 5.6.) except that the [^{14}C] nicotinamide was replaced by [^{3}H]thymidine. The control embryos were counted (★——★). The test embryos had their radioactive content determined directly (△——△) (See legend Figure 5.5.).


100
80
60
40
20
% germination
10
8
6
4
2
CPM [³H] thymidine incorporated (x10⁻³)
0
4
8
12
16
20
24
Time (hours)

fluorescent when irradiated at 260nM. After 1 hour of germination in 3-aminobenzamide, a highly fluorescent spot could be seen on the filter paper. Attempts to quantitate the uptake of 3-aminobenzamide using a fluorimeter failed. The background fluorescence was always high and at a low level of detection needed, the fluorimeter was unstable. The conclusion must be that the three inhibitors enter the embryo cells. At this point it can be assumed that inhibition of the ADP-ribose transferase has no detectable effect on germination in the wheat embryo. Such an assumption is weakened somewhat by the fact that a decrease in protein ADP-ribosylation must have occurred if the ADP-ribose transferase is inhibited. No quantitative or qualitative assay for ADPR/protein was available when this work was carried out and so the direct effects of the nicotinamide, thymidine and 3-aminobenzamide on ADP-ribosylation could be determined. This lack of an effect on germination by these inhibitors is perhaps not surprising in the light of recent work in this laboratory where L1210 mouse tumor cells have been grown in 2mM 3-aminobenzamide for ten months without affecting their growth rate(Whish, 1982 personal communication). It therefore seems that ADP-ribose transferase does not play a direct role in cell growth and division.

An interesting observation in these experiments is that germination was unaffected by a high concentration of

thymidine. In animal cells 2mM thymidine is used in the classical double thymidine block for cell synchronisation (Mitchison, 1977). That is, DNA synthesis is inhibited by thymidine through its action on d CTP synthesis. However, as seen in this work, high levels of thymidine had no effect on either the rate of germination or the percentage viability of the embryos. Thus it seems that in the wheat embryo, DNA synthesis is unaffected by thymidine concentrations which would normally completely inhibit DNA synthesis in animal cells. Furthermore Whitby et al (1979) showed that thymidine was a potent inhibitor of ADP-ribosylation in isolated nuclei and yet in vivo it had no effect on germination. In the absence of a suitable quantitative assay for protein ADP-ribosylation, the above results are good evidence that germination studies using wheat embryos in the presence of ADP-ribose transferase inhibitors is not a fruitful approach to the analysis of the biological function of ADP-riboslyation in seeds.

A final series of experiments were carried out to see if an alternative way of studying germination might exist.

As has been seen earlier, plants seem to behave abnormally (relative to animal cells) in their resistance to thymidine inhibition of DNA synthesis.

The next section describes attempts to manipulate germination using inhibitors of macromolecular synthesis in an attempt to perturb ADP-ribosylation and/or germination.

5.3.2. Polynucleotide Synthesis Inhibitors

For these experiments two compounds, hydroxyurea and cordycepin (3'deoxyadenosine), were used. Hydroxyurea was chosen because it is a well characterised, widely used (Yarbro, 1967) inhibitor of replicative DNA synthesis (not repair DNA synthesis).

The conditions used for these germination experiments were identical to those outlined in Section 5.3.1. except that 5mM hydroxyurea was present in the germinating medium. As can be seen from Figure 5.8 the high level of hydroxyurea has only a small effect on the rate of germination. It is apparent that the rates of the hydroxyurea and the control experiments are linear for the first eight hours. A simple comparison indicates that in 8hrs the control embryos are 50% germinated whilst the hydroxyurea treated embryos lag by just 8% at 42%. After the final 24 hours germination in each case is 93% for the control and 83% for the hydroxyurea. If the embryos are left longer than 24 hours, the difference between the control and hydroxyurea becomes even less significant. When nuclei were isolated from the hydroxyurea and control embryos no difference in NAD incorporation could be detected. That is, the rate of ADP-ribosylation for both types of embryos was typical of a normal incorporation curve (see Figure 5.9).

The conclusion for the hydroxyurea work must be that,

Figure 5.8.

Time course for germination of wheat embryos, the effect of 5mM hydroxyurea

Embryos were germinated under standard conditions (as described in Section 3.2.1. and Figure 5.1.). The experimental system is the same as described in Figure 5.1. except that one germination dish (the test) contained 5mM hydroxyurea (▼——▼).

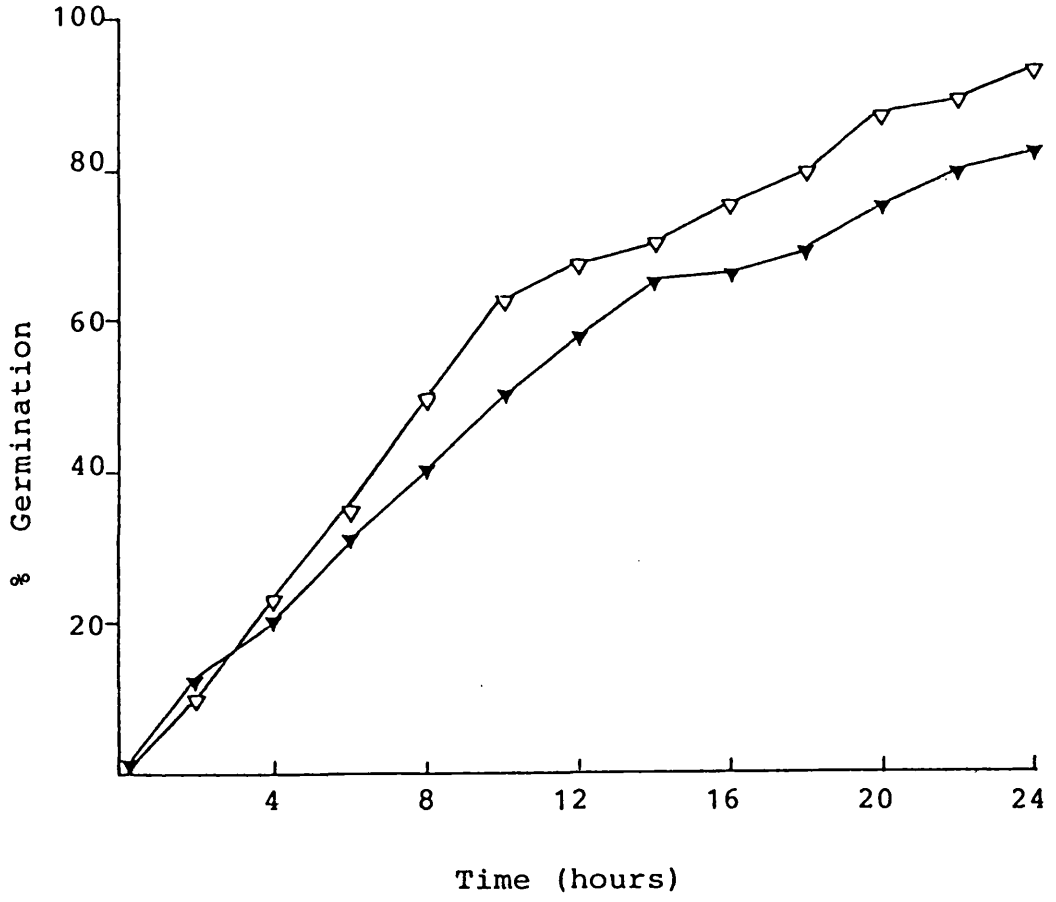

100
80
60
40
20
% Germination
4
8
12
16
20
24
Time (hours)

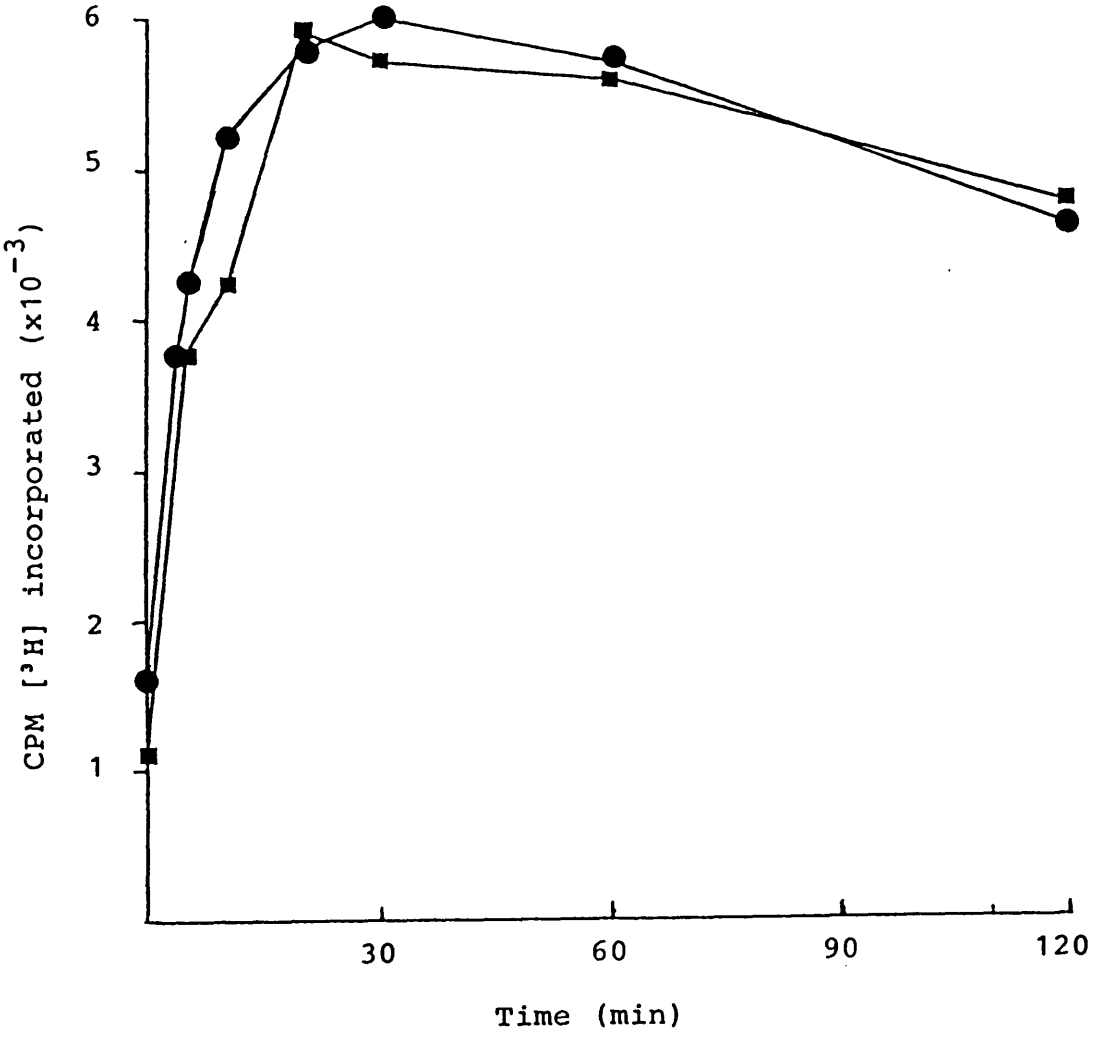
Figure 5.9.

Effect of hydroxyurea (5mM) on poly(ADP-ribose) activity in isolated wheat nuclei.

100 embryos were treated with 5mM hydroxyurea for 18 hours under standard germinating conditions (Section 3.2.1. and Figure 5.1.). Control embryos were also germinated but without hydroxyurea. After 18 hours germination, the test and control embryos were washed and nuclei isolated from them (Section 3.2.2.). These nuclei were then incubated under standard conditions with radioactive NAD (Section 4.1.3. and Figure 4.2.) and the rate of incorporation of NAD measured.

●——● nuclei from normal embryos

■——■ nuclei from hydroxyurea treated embryos


6
5
4
3
2
1
CPM [³H] incorporated (x10⁻³)
30
60
90
120
Time (min)

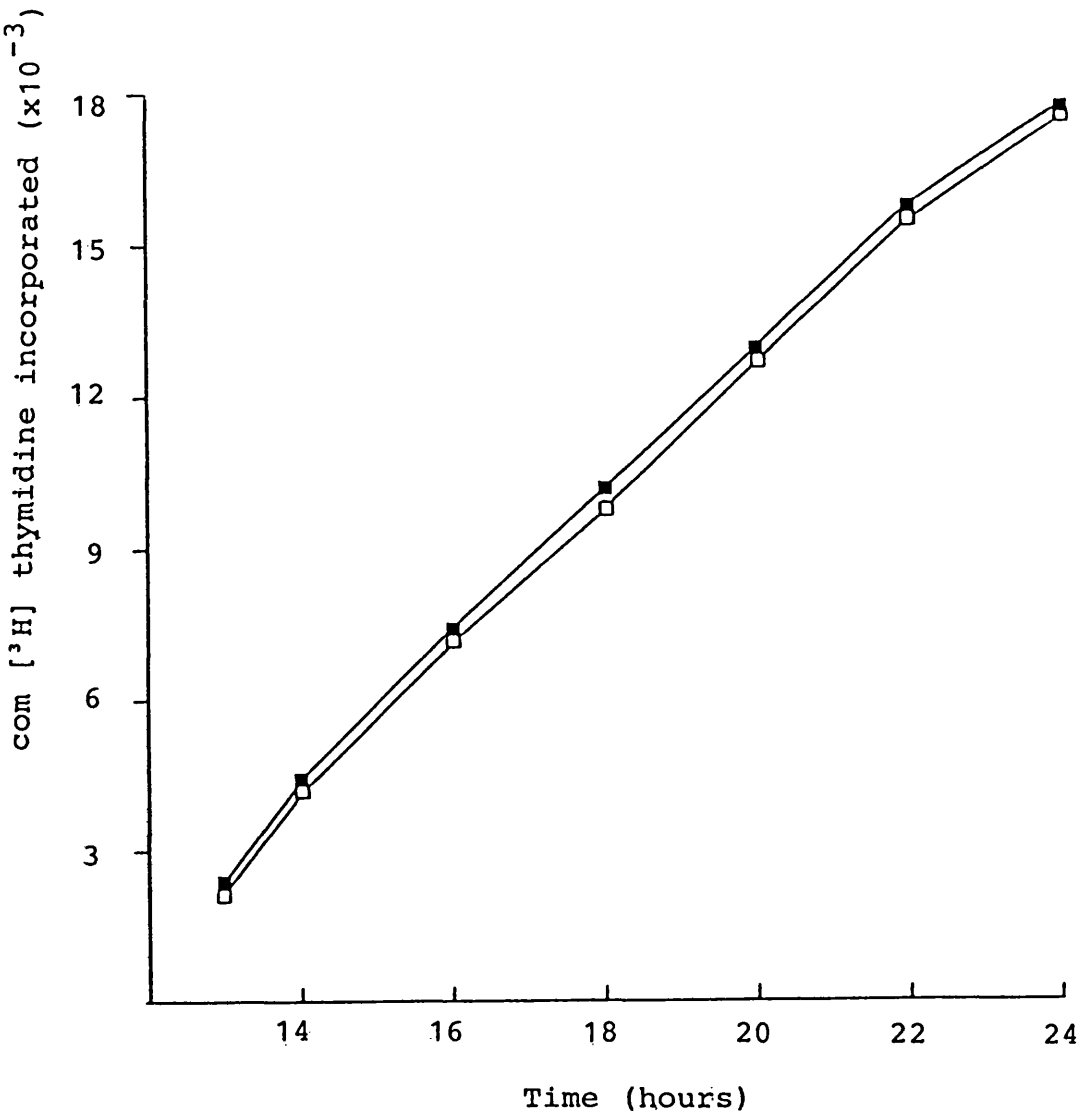
once again, plants behave abnormally compared to animal cells, in that DNA synthesis is unaffected by hydroxyurea. This conclusion was supported by a series of experiments where the rate of DNA synthesis in embryos ± hydroxyurea was analysed using [^{3}H]thymidine uptake (see Figure 5.10). The experimental design was that described in Section 5.3.1. for hydroxyurea except that in both control and hydroxyurea treated, germinations, tritiated thymidine was present at a concentration of 5μCi/ml of germination medium. At each time point in Figure 5.10, 2 embryos were removed and homogenised in 20% TCA. The homogenate was left on ice for an hour and the whole washed over a Whatman GF/C filter (as described for acid precipitation in Section 3.2.4.) and counted. As can be seen from Figure 5.10, the rate of thymidine incorporation into embryo DNA is unaffected by hydroxyurea. This supports the conclusion drawn above that in wheat embryos DNA replication synthesis is unaffected by hydroxyurea. This was a disappointing result because there is now no simple way of inhibiting DNA replication in the embryo in order to study more closely DNA repair synthesis.

As stated at the beginning of this section experiments were also attempted with cordycepin. This is a potent inhibitor of polynucleotide synthesis in animal systems (Suhadolnik, 1970) because it is rapidly phosphorylated to cordycepin triphosphate (3-deoxy adenosine triphosphate)

Figure 5.10.

Effect of hydroxyurea 5mM on DNA synthesis in germinating wheat embryos using [^{3}H]thymidine

Embryos were germinated under standard conditions (Section 3.2.1. and Figure 5.1.). Both control and test germinations contained 5μCi/ml [^{3}H]thymidine. Additionally, the test (□——□) also contained 5mM hydroxyurea. At each time point, two embryos were removed, washed, homogenised and their acid-insoluble radioactivity determinated (Section 3.2.4.).


com [³H] thymidine incorporated (x10⁻³)
18
15
12
9
6
3
14
16
18
20
22
24
Time (hours)

which is then recognised by both the RNA and DNA synthesising systems. Thus the 3'-deoxyadenosine monophosphate is incorporated into DNA and RNA. However, once incorporated no further chain extension can occur because the 3-deoxy prevents formation of further 3'-5' phosphodiester linkages. The germination experiments were carried out as described in Section 5.3.1. except that the test embryos germinating medium contained the relevant isotopic polynucleotide precursor plus 100µg/ml cordycepin.

As can be seen from Figure 5.11, cordycepin has quite a dramatic effect on polynucleotide and DNA synthesis (see Figures 5.11 and 5.12). Thymidine incorporation drops to about 20% of the control. The inhibition is even greater when cordycepin is present at 100µg/ml germination medium (Figure 5.12).

Figure 5.12 illustrates this point and here the data indicate that thymidine incorporation has dropped by approximately 90% of the control level.

The effect of cordycepin on RNA synthesis was then investigated. The problem here is that labelled uridine is incorporated only at rather low levels into RNA when compared to thymidine incorporation into DNA. The reasons for this are not clear. Meaningful counts can be obtained, but only at relatively high concentrations of isotope. However such experiments become prohibitively expensive, especially if such experiments have to be repeated a number of times. Uridine labelling was only

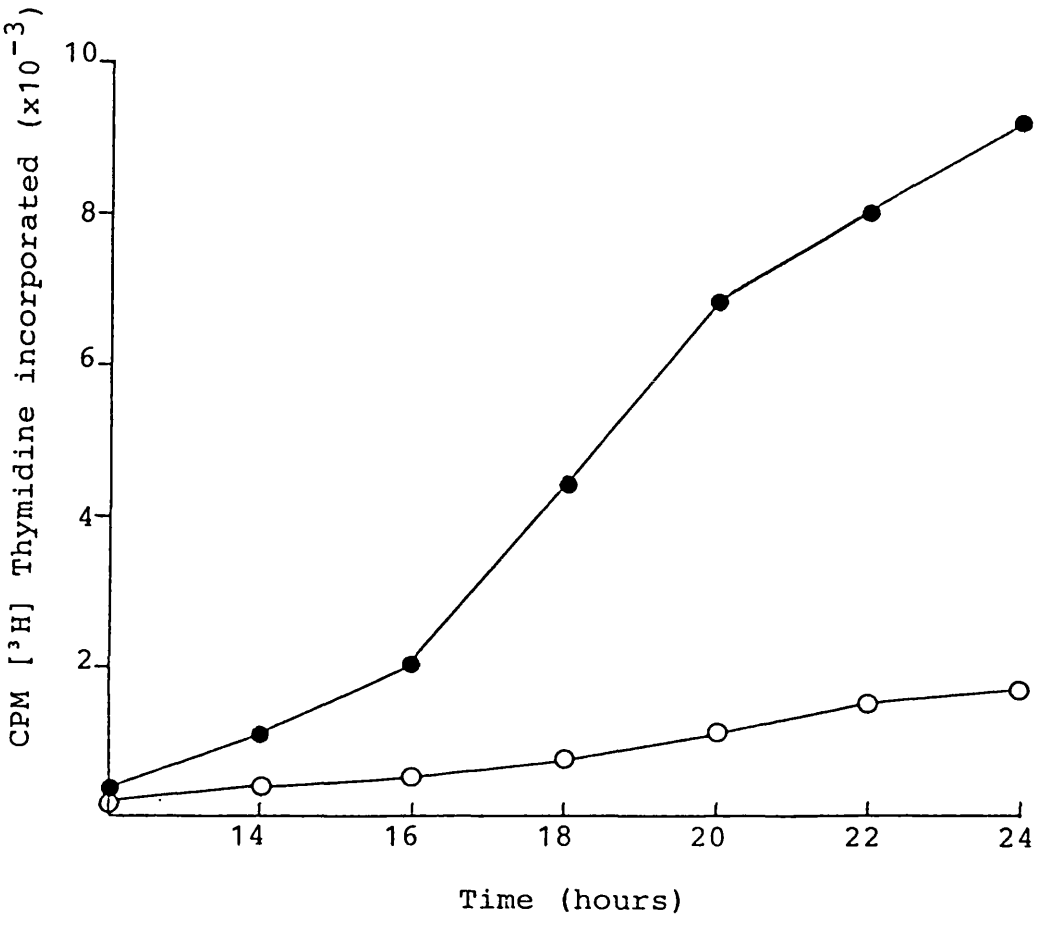

CPM [³H] Thymidine incorporated (x10⁻³)
10
8
6
4
2
14
16
18
20
22
24
Time (hours)

Figure 5.12.

The effect of cordycepin on DNA synthesis in germinating wheat embryos

The conditions of this experiment were almost identical to those described in the legend to Figure 5.11. The only difference being that in the test, the cordycepin was present at 100μgm/ml.

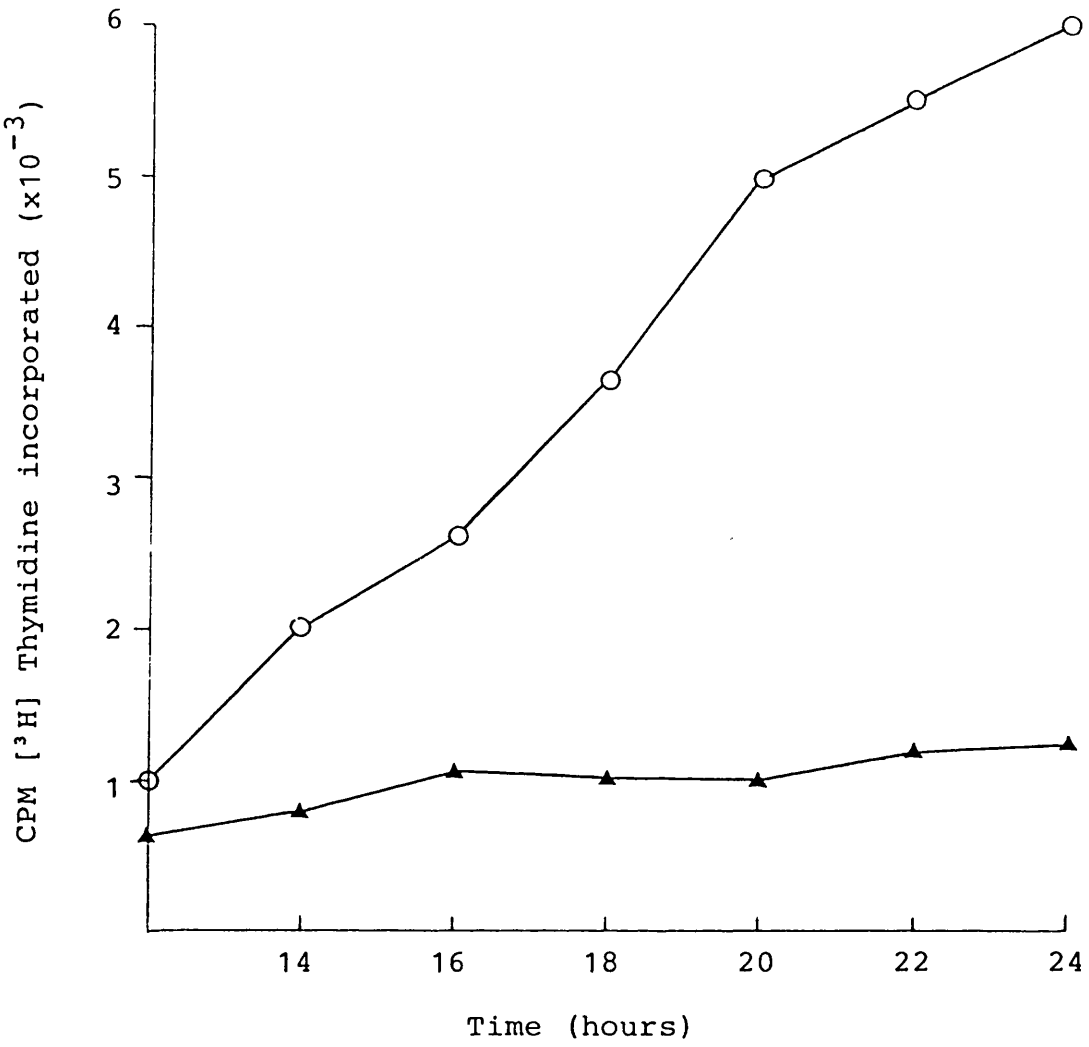

CPM [³H] Thymidine incorporated (x10⁻³)
6
5
4
3
2
1
14
16
18
20
22
24
Time (hours)

Figure 5.13.

The effect of cordycepin on DNA and RNA synthesis in germinating wheat embryos

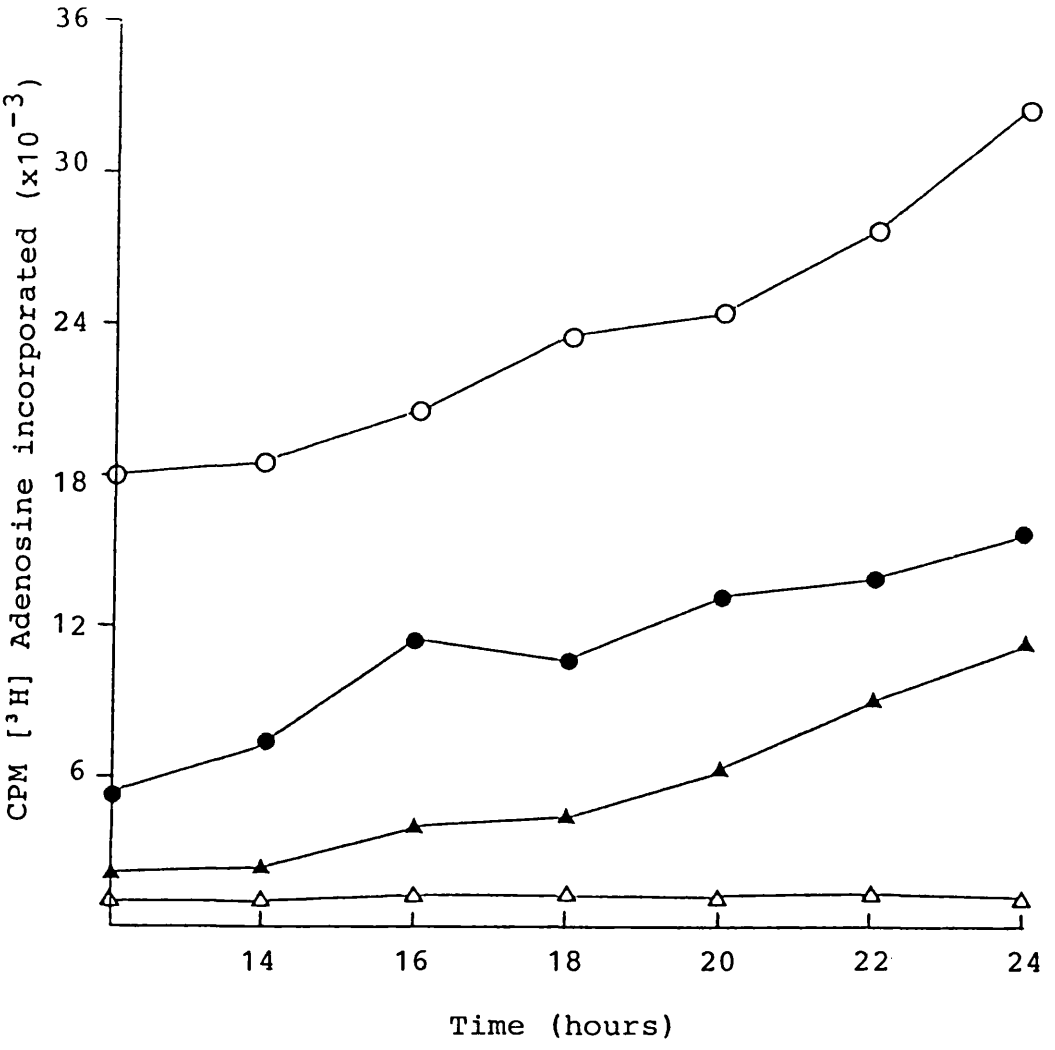
Embryos were germinated under standard conditions (Section 3.2.1. and Figure 5.1.). Both the control and test germination contained 5μCi/ml [^{3}H]adenosine and the test additionally contained 50μg/ml cordycepin (see Section 5.3.2. and page 102). At the times indicated, embryos were removed and homogenised. The DNA and RNA, and DNA alone were assayed.

○———○ control, total polynucleotide synthesis (DNA + RNA).

▲———▲ control, DNA synthesis alone.

●———● test, plus cordycepin (50μgm/ml), total polynucleotide (DNA + RNA).

△———△ test, plus cordycepin (50μgm/ml), DNA synthesis alone.


36
30
24
18
12
6
CPM [³H] Adenosine incorporated (x10⁻³)
14
16
18
20
22
24
Time (hours)

used in this work for the ultracentrifugation analysis of ADP-ribosylated proteins (see Section 3.2.6.). An alternative approach is to use ^{3}H-adenosine and (a) acid precipitate the total polynucleotides then (b) base treat an identical aliquot (0.1M NaOH at 37°C overnight (Goebel et al 1977) with subsequent acid precipitation one simply substracts (b) from (a) to give the cpm incorporated into RNA. (b) gives the incorporation into DNA because the base hydrolyses all the cellular RNA.

Over a period of 24 hours of germination in the presence of cordycepin (Figure 5.13) DNA synthesis is closed to zero (△———△). Thus curve ●———● almost completely represents RNA synthesis in the presence of cordycepin. Thus this inhibitor does not stop RNA synthesis. In fact a visual comparison between the control DNA and RNA synthesis (O———O) and the cordycepin DNA + RNA synthesis (●———●) shows that there is really only a small difference between them. It must be emphasised that quantitative analysis of such curves is not advisable because each curve represents a different, germinating set of embryos, and whilst comparisons within a culture of embryos is meaningful, comparison between cultures is not.

At this stage it was realised that the use of cordycepin was not going to be a fruitful approach to ADP-ribosylation in vivo (see conclusion). A look at the results in detail illustrates why.

The data obtained with cordycepin is quite complicated when analysed in depth. The 3'd ATP will not only affect polynucleotide synthesis as described earlier, but will also perhaps have a profound effect on NAD synthesis. This will occur because NAD pyrophosphorylase has a fairly wide specificity for nucleoside triphosphatese (Morton, 1958). Thus it might be expected that the 3'dATP will be combined with NMN to form the 3'deoxy adenosine analogue of NAD (N3'dAD) by the pyrophosphate. Suhadolnik et al.,(1977) has shown that the 2'deoxy adenosine analogue of NAD is accepted as a substrate by (ADP-ribose)transferase. Thus it is likely that N 3'dAD is also a substrate for ADP-ribose transferase. The N 3'dAd might inhibit both branched chain structures (Miwa et al., 1979) and chain elogation. This is of course supposition, but it can be seen that the earlier thought, that cordycepin might have a single specific effect on the embryos, is probably wrong. It must be concluded that cordycepin will have at least two profound effects and probably many more. It was for this reason that no further work was carried out using cordycepin.

The last two sections involving the use of inhibitors as probes for germination and ADP-ribosylation studies have shown that for the reasons given such experiments are inappropriate.

The next sections detail attempts to study ADP-ribosylation of nuclear protein directly in the intact embryos.

5.4. Determination of Protein ADP-Ribosylation in vivo

As stated earlier (Section 5.1) the wheat embryo, like the animal cell is not permeable to phosphorylated nucleosides. Furthermore the embryo is not amenable to permeabilisation. Therefore it is not possible to use a radiolabelled specific substrate (e.g. NAD) for the ADP-ribose transferase in vivo. The precursor of choice is [^{3}H] adenosine because it is rapidly taken up by the cell and phosphorylated to ATP. The disadvantage of adenosine is that the labelled ATP will not only be incorporated into NAD but will also be rapidly taken up into DNA and RNA. Therefore any analytical system must be capable of separating large amounts of radioactive DNA and RNA from small amounts of ADP-ribosylated protein. A method was developed in this laboratory by Surowy (1981) who used caesium chloride-urea isopycnic ultracentrifugation to quantitatively separate polynucleotides from proteins.

Poly (ADP-ribose) or ADP-ribose will be covalently linked to proteins. The buoyant density of these proteins is sufficient to allow protein/ADPR complexes to band at or near the top of a caesium chloride gradient (in a low density) whereas the DNA and RNA will move to near the bottom of the gradient at a high density.

The sample is dissolved in 10M urea containing 0.1M sodium acetate pH 6.5. After mixing well, an equal volume of 100% caesium chloride (w/v) in 0.1M sodium acetate pH 6.5 is added. The initial caesium chloride density is 1.55gm/cm^3. After centrifugation in a Beckman Sw 50.1 rotor at 45,000 rpm for 72 hours at 15°C, the resulting gradient has a density of 1.25 gm/cm^3 at the top and 1.9 gm/cm^3 at the bottom (see Figure 5.14). The initial 10M urea is essential in this method because it dissociates most of the DNA-protein and RNA – protein complexes so that when caesium chloride is added, even though the urea concentration drops to 5M, reassociation does not appear to occur. The following detailed experiments show this.

Embryos were germinated under standard conditions (Section 5.3.1.) except that tritium-labelled thymidine, or [^{3}H]leucine was present at a concentration of 5µCi/ml and [^{3}H]uridine was present at a concentration of 50µCi/ml germination medium. The embryos were collected after 24 hours germination and the extracellular isotope washed off with germinating medium. Disruption and solubilisation of the embryos was achieved by homogenisation of 10 embryos per 0.5ml of 10M urea/0.1M sodium acetate pH 6.5 in a tight fitting Potter-Elvjehm teflon glass homogeniser. A light centrifugation step of 1000xg for ten minutes removed urea-insoluble chaff and other material. The 10M urea solution was then diluted 1:1 with 100% (w/v) caesium chloride 0.1M sodium acetate pH 6.5, the resulting solution being

centrifuged at 45,000 rpm for 72 hours at 15°C. The gradients were fractionated by inserting a needle into the bottom of the tube and fractionating the contents with the use of pump and fraction collecter. The rate of collection was not allowed to exceed 1 gradient per 10 minutes, otherwise mixing of the tube contents occurred. Suitable aliquots of each tube were then acid precipitated onto Whatman 3mm discs (See Section 3.2.4.).

As can be seen from Figure 5.14 DNA (thymidine labelled) sediments to the bottom of the gradient. Most importantly, no DNA is present at the top of the tube.

Figure 5.15 shows that uridine labelled RNA also sediments to the highest density regions of the gradient. Visual inspection of the graph indicates that fractions 10 and 11 (top) might contain some RNA. A detailed analysis (Chapter 6) showed that 5-10% of RNA remains at the top of the gradient. Figure 5.16 shows the gradient profile when [^{3}H] leucine is used to label the proteins. In this case most of the protein is at the top, or in the top 1/3rd of the gradient. These controls show that DNA, RNA and protein separate well on this caesium chloride urea gradient. A final control was run using isolated wheat embryo nuclei which had been labelled with radioactive NAD, using the standard nuclear labelling procedure (see Section 4.1.3.). Figure 5.17 indicates that all of the acid insoluble material bands at the top of the gradient.

Figure 5.14.

Analysis of [^{3}H]thymidine labelled embryos on CsCl-urea density gradient centrifugation

Embryos (100) were germinated under standard conditions (Section 3.2.1. and Figure 5.1.). They also contained 5μCi/ml [^{3}H]thymidine. The germination was continued for 24 hours, when the embryos were washed well in germination buffer (Section 5.4.) and homogenised in 8M urea in 0.1M sodium acetate, pH 6.5. They were lightly centrifuged (2,000 x g) to remove debris, and an equal volume of 100% CsCl (w/v) in 0.1M sodium acetate pH 6.5 was added. The whole was mixed well and then centrifuged to equilibrium (72 hours) at 45,000 rpm, 15°C in a Beckman 50.1 rotor. The gradient was fractionated and the radioisotope determined (Section 3.2.4.) (■———■). Fraction density was also determined (See Section 3.2.6.) (□———□).

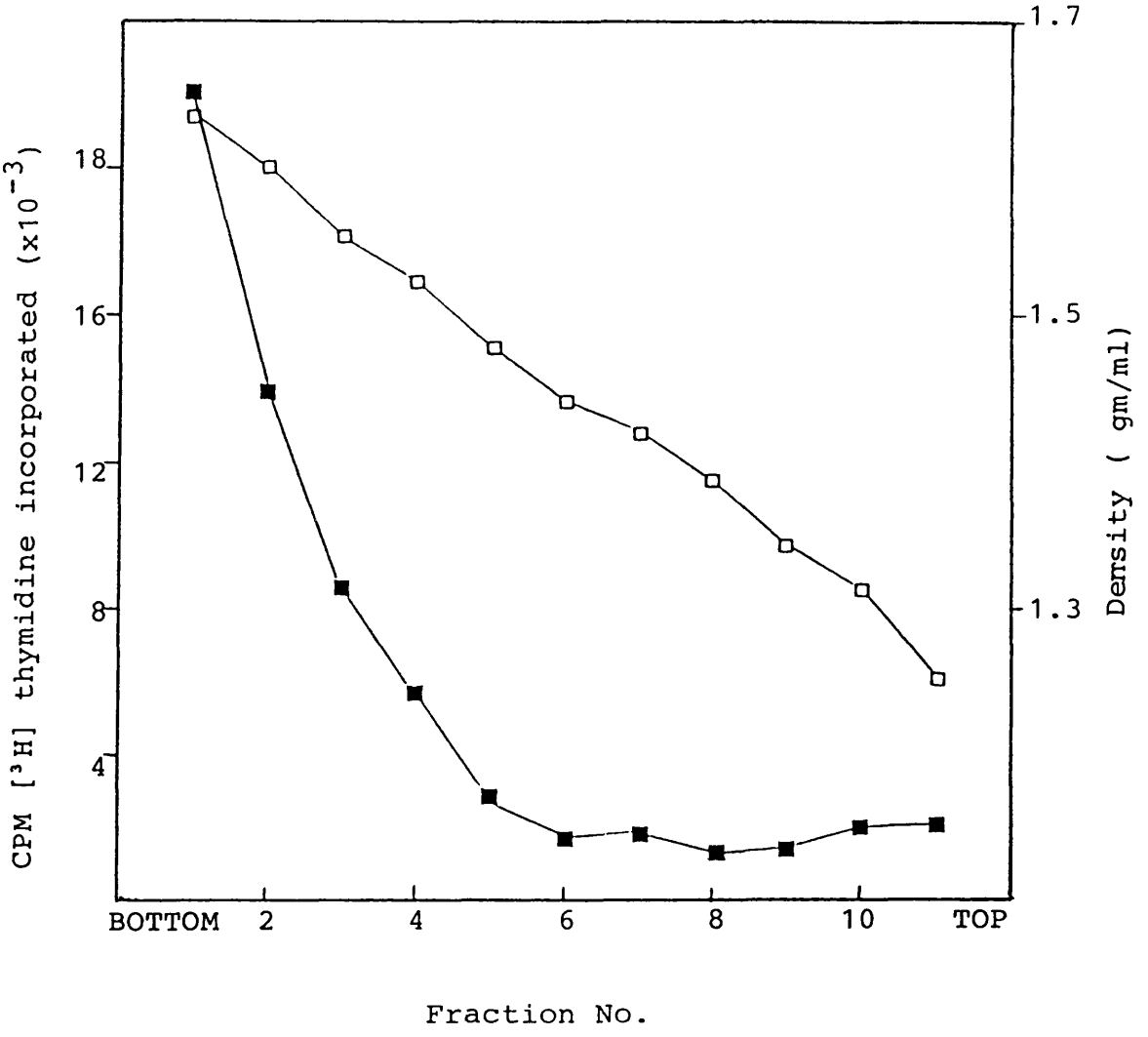

CPM [³H] thymidine incorporated (x10⁻³)
18
16
12
8
4
1.7
1.5
1.3
Density (gm/ml)
BOTTOM
2
4
6
8
10
TOP
Fraction No.

Figure 5.15.

Caesium chloride density gradient, centrifugation of uridine (RNA) labelled cells

Embryos were germinated under standard conditions (Section 3.2.1. and Figure 5.14). Conditions were identical to those described in Figure 5.14 except that the uridine (5μCi/ml) replaced the [^{3}H]thymidine. The ultra centrifugation and analysis of the gradients was identical to Figure 5.14.

■——■ radioactivity incorporated ([^{3}H]UMP in RNA)

□——□ density (gm/ml)

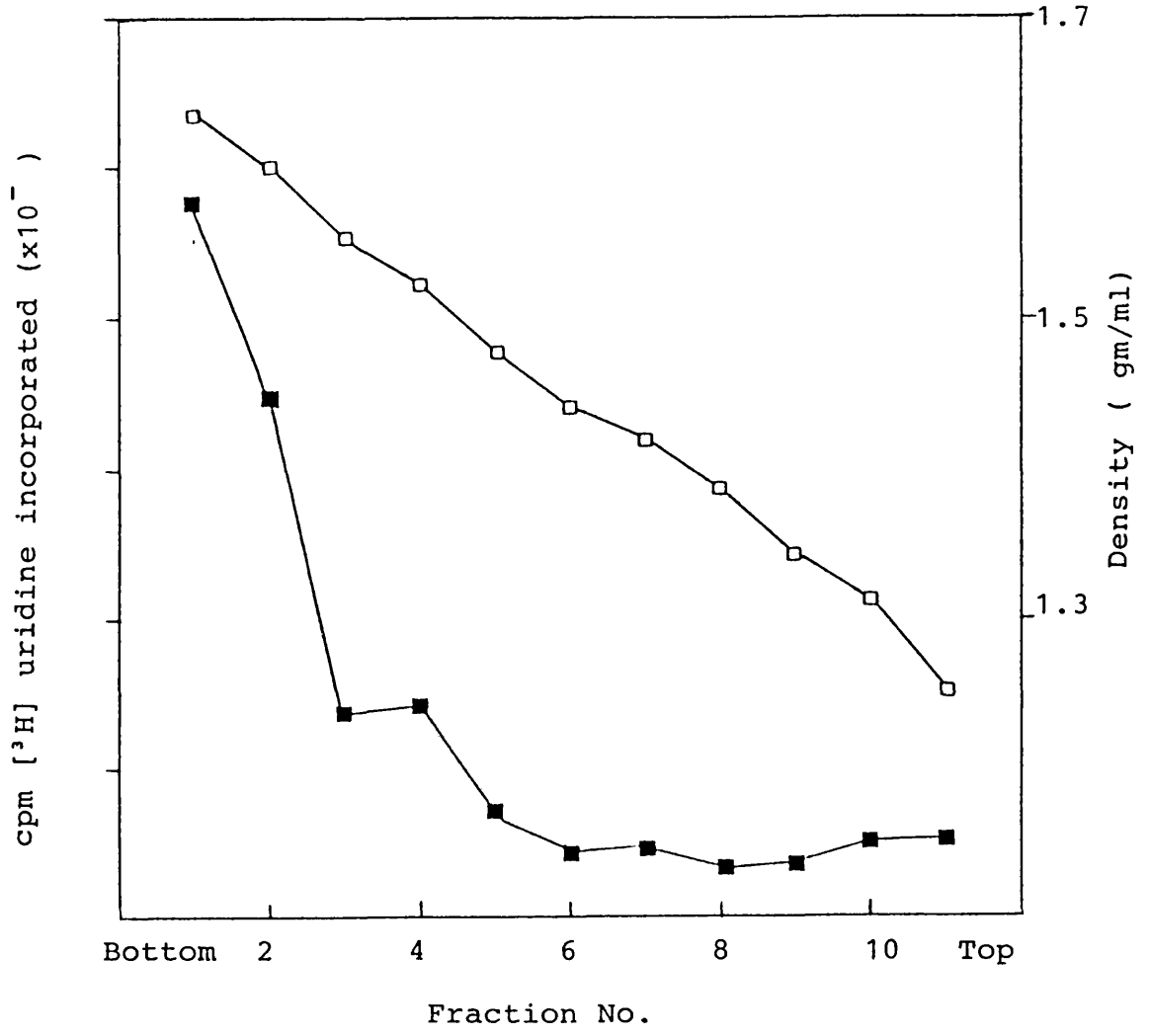

cpm [³H] uridine incorporated (x10⁻)
Density (gm/ml)
1.7
1.5
1.3
Bottom
2
4
6
8
10
Top
Fraction No.

Figure 5.16

Caesium chloride density gradient centrifugation, separation of proteins from polynucleotides

Embryos were germinated under standard conditions (Section 3.2.1. and Figure 5.14). Conditions were identical to those in Figure 5.14 except that [^{3}H] leucine replace the [^{3}H]thymidine. The ultracentrifugation and subsequent analysis of the gradient was identical to that described in Section 5.4 and Figure 5.14.

O———O radioactivity incorporated into protein ([^{3}H]leucine)

▲———▲ density gm/ml

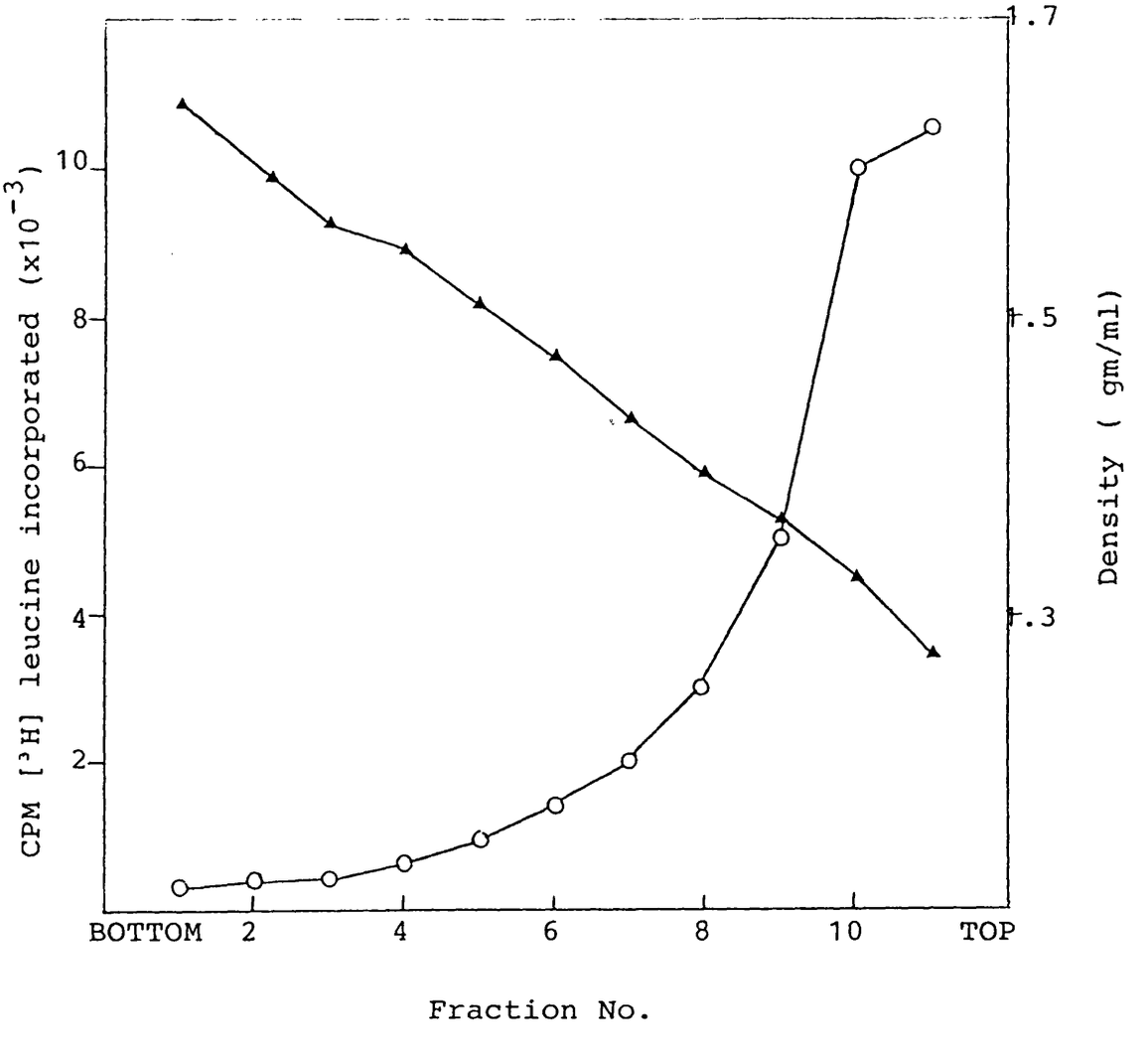

CPM [³H] leucine incorporated (x10⁻³)
10
8
6
4
2
1.7
1.5
1.3
Density (gm/ml)
BOTTOM
2
4
6
8
10
TOP
Fraction No.

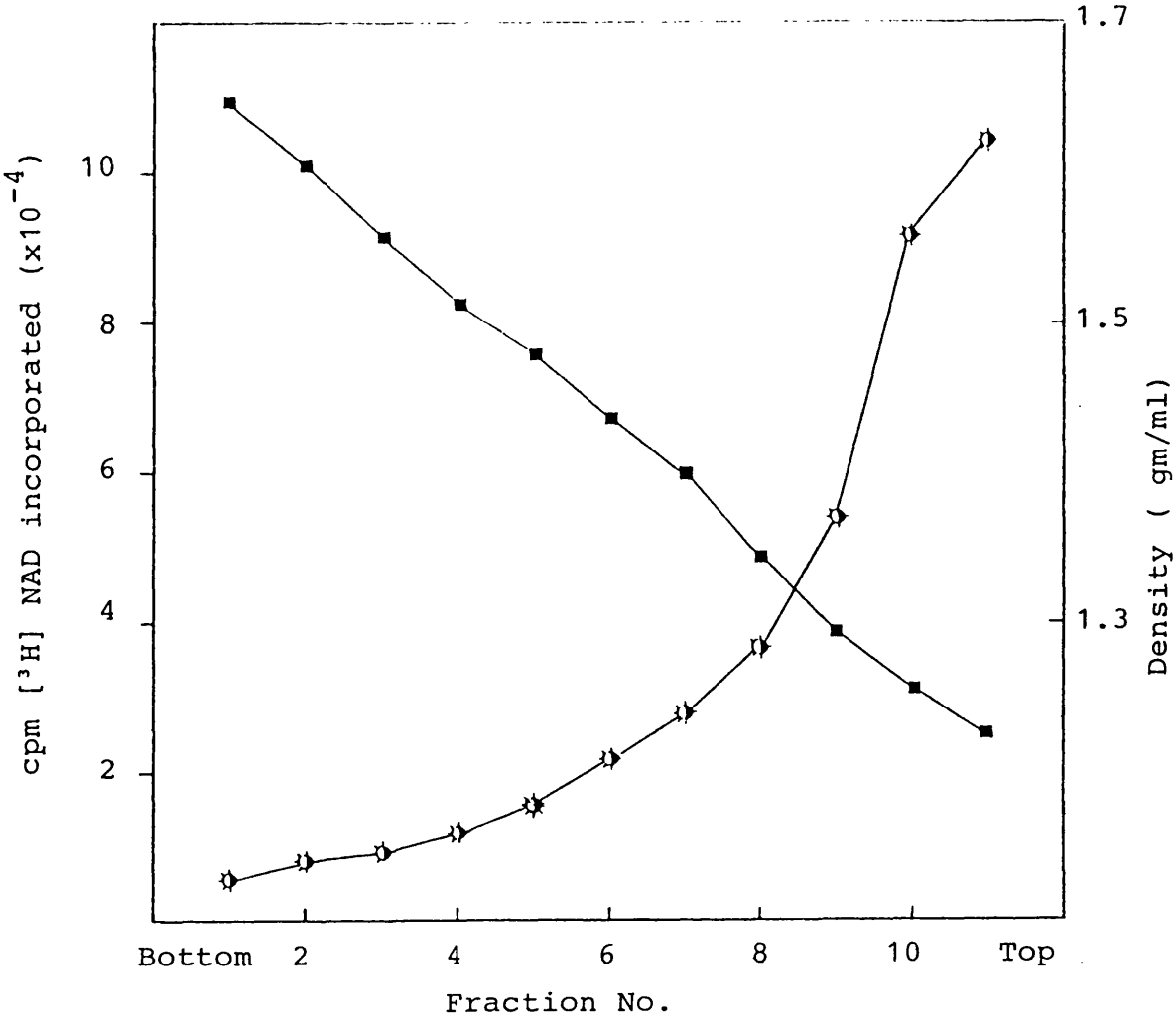
Figure 5.17

Caesium chloride density gradient centrifugation of NAD labelled isolated nuclei

Nuclei were isolated and labelled with radioactive NAD as described in Section 3.2.2. and Section 4.1.3. respectively. The labelled nuclei were added 2.5ml 8M urea/0.1M sodium acetate pH 6.5 followed by 2.5ml 100% CsCl (w/v) in 0.1M sodium acetate pH 6.5. The mixture was centrifuged to equilibrium (72 hours) at 45,000 rpm and 15°C (Section 3.2.6.). The acid-insoluble radioactivity was then determined throughout the gradient (Section 3.2.4.)

◐——◑ [^{3}H]NAD labelled nuclei

■——■ density gm/ml


cpm [³H] NAD incorporated (x10⁻⁴)
10
8
6
4
2
1.7
1.5
1.3
Density (gm/ml)
Bottom
2
4
6
8
10
Top
Fraction No.

Having shown that the caesium chloride urea gradient gave good separation of polynucleotides and ADP-ribosylated proteins in wheat embryos, experiments were then designed to analyse ADP-ribosylated proteins in vivo.

Once again the germination procedure was the standard one outlined in Section 5.3.1., except that radioactive adenosine was added to the germinating medium at a concentration of 20μCi/ml.

Figure 5.18 shows the typical profile which is obtained when embryos are labelled with adenosine and then washed, homogenised and ultracentrifuged as described earlier in this section for thymidine uptake. The results are also highly reproducible in that 28% of the total acid insoluble ^{3}H-adenosine is in the protein fraction of the gradient and the remainder (from fraction 6 downwards) is at the DNA/RNA density. It is worth noting that in this gradient, the DNA/RNA and adenosine labelled proteins are well separated. As suggested earlier, the gradient profiles were highly reproducible from experiment to experiment such that for detailed analysis of this material the top 2mls (fraction 8 and above) of each gradient was removed with a syringe and bent needle. The gradients were not routinely fractionated but checks were frequently run to show that the protein fraction of the gradient did not contain DNA and RNA.

It can be concluded that the caesium chloride urea

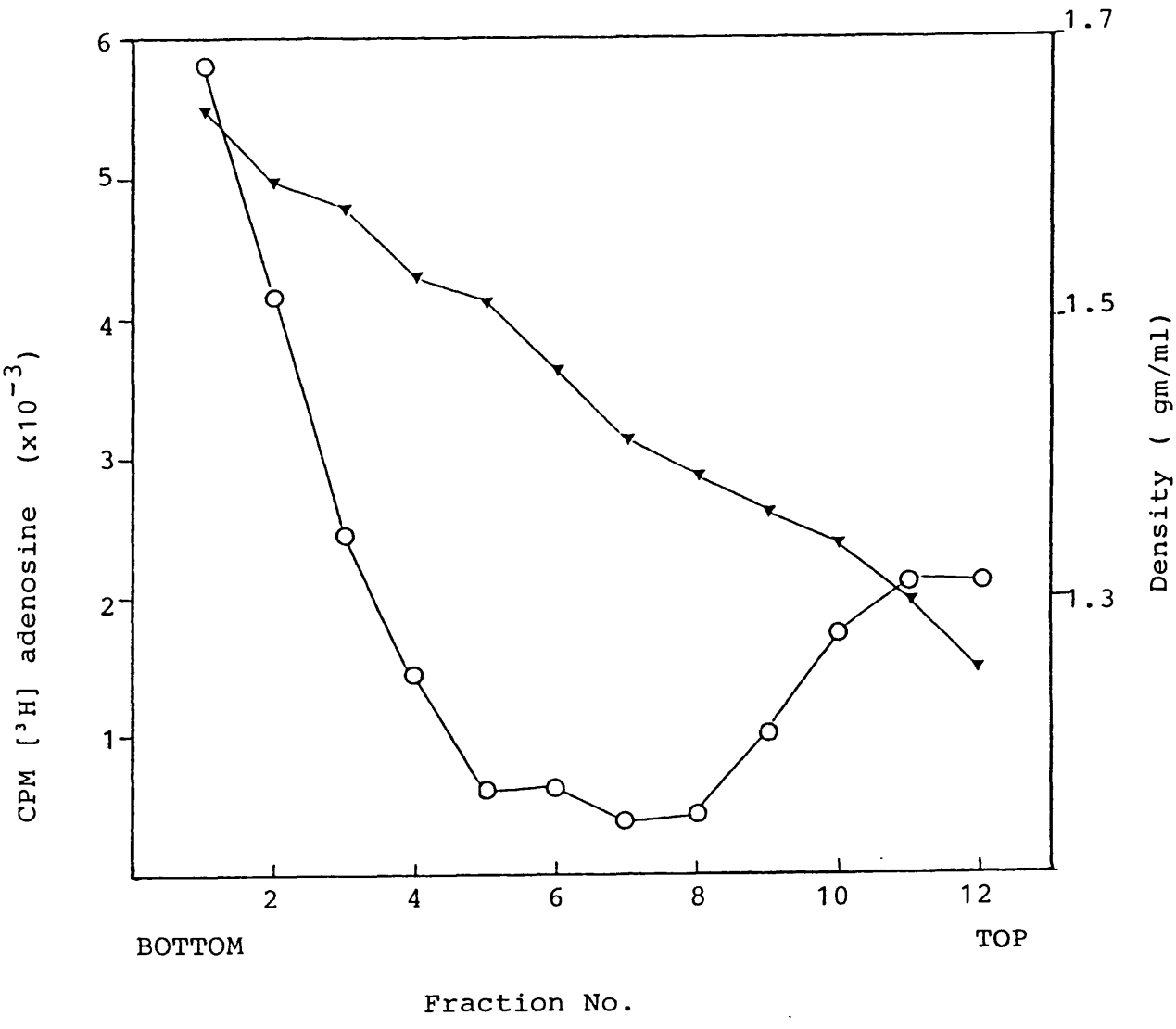
Figure 5.18

Caesium chloride density gradient centrifugation, separation of protein bound ADP-ribose and protein bound adenosine from DNA and RNA

Embryos were germinated under standard conditions (Section 3.2.1. and Figure 5.14). The conditions of the experiments was identical to that described in Figure 5.14 except that [^{3}H]adenosine (5μCi/ml, 20Ci/mmole) replaced the [^{3}H]thymidine. The ultracentrifugation and analysis of the gradient was identical to that described in Figure 5.14.

O———O [^{3}H]adenosine incorporated

▼———▼ density gm/ml


CPM [³H] adenosine (x10⁻³)
Density (gm/ml)
1.7
1.5
1.3
6
5
4
3
2
1
2
4
6
8
10
12
BOTTOM
TOP
Fraction No.

gradient is a useful and reproducible method for separating other polynucleotides from "adenosine containing proteins". The next chapter details experiments which were carried out to characterise the nature of the adenosine moiety of these protein complexes, as well as attempts to characterise the proteins themselves.

CHAPTER 6

6.1. Characterisation of ^{3}H-Adenosine/Protein Complex Isolated Using Caesium Chloride-Urea Gradient Ultracentrifugation

The starting material was always prepared as outlined in Section 5.4 as stated in there, the top two millilitres of each gradient tube was used for the work described here. The adenosine labelled proteins when removed from the gradient centrifuge tubes were, of course, already dissolved in caesium chloride and 4M urea. The quickest and most nearly quantitative method for removing the salt and urea was to acid precipitate each one millilitre of adenosine protein material with 5 millilitres of 20% TCA. This was then mixed well and kept at 0°C (on ice) for four hours, with subsequent centrifugation at 100,000g for 60 minutes in a Beckman $SW_{50.1}$ rotor. The supernatant was discarded after being filtered through a Whatman GF/C filter which was then dried and counted (Section 3.2.4.) A direct check was therefore kept on any losses of acid insoluble material in these supernatants. Usually less than 2% of the acid insoluble counts were found on the filter, the rest were always present in the pellet. Any TCA was removed from the pellet by washing it with 5millilitres of cold 75% ethanol made to 1M in acetic acid. This suspension was briefly centrifuged at 1000g at 4°C in a bench centrifuge. The superantant was discarded after pouring it through a Whatman GF/C filter as

mentioned earlier. Once again the losses here were always less than 2% of the total acid insoluble material. This "adenosine-pellet" was used for all subsequent analyses.

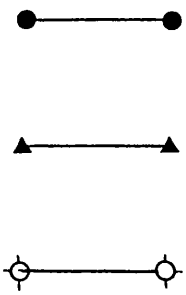
The first procedure carried out was to digest the adenosine pellet exhaustively with sodium hydroxide (37°C overnight). This hydrolyses all the RNA (if present) to 3'nucleoside monophosphates. All base sensitive ADPR/protein and poly ADPR/protein linkages are broken (Bredhorst et al., 1978). The ADP-ribose derived from the former is subsequently hydrolysed to 5'AMP and compound X (Goebel et al, 1977) in a ratio about 9:1 5'AMP: compound X. The poly (ADP-ribose) freed from proteins is unaffected by base under these conditions. DNA, if present, is also unaffected by the sodium hydroxide.

Figure 6.1 shows that the base treatment does not render the adenosine pellet completely acid soluble. After 6 hours (and 24 hours) about 40% of the acid insoluble adenosine is still present. This is likely to be either long polymer or DNA or both (see later, this Section). It may also be due to base resistant ADPR/protein covalently linked complexes. After 18 hours of base treatment, the pH of the above base digest was adjusted to between pH 5 and 7 with 6M acetic acid, and the whole applied to a 5mm x 1cm amino ethyl-cellulose column (see Materials and Methods Section 3.2.8). The column was first washed with 6M acetic acid. This removed all nucleoside monophosphates. The acetic acid was removed with a distilled water wash,

Figure 6.1.

Time course of hydrolysis of [^{3}H]adenosine-labelled material with sodium hydroxide and ethylamine

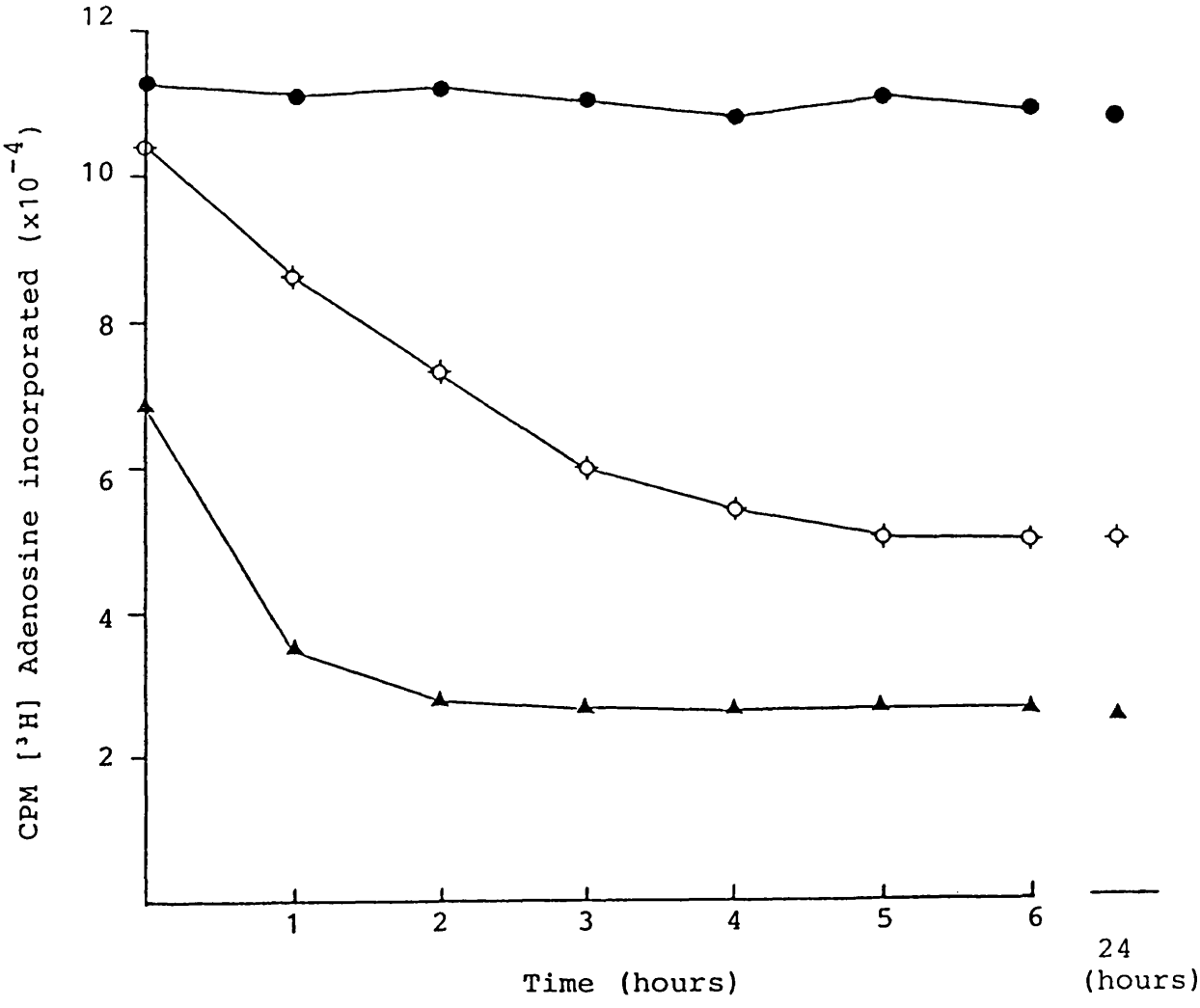
The adenosine pellet from one gradient (page 109-110) was suspended (dissolved) in 0.2M NaOH (5 ml) and incubated at 37°C. At various times, aliquots were removed and acid precipitated and counted (Section 3.2.4.).



shows the curve obtained with incubation at neutral pH

Indicates the hydrolysis of acid-insoluble material with NaOH

Indicates the hydrolysis of acid-insoluble material with 1M ethylamine and no NaOH.


12
10
8
6
4
2
CPM [³H] Adenosine incorporated (x10⁻⁴)
1
2
3
4
5
6
24
(hours)
Time (hours)

The final wash was with 1M ethylamine (Whish's group, 1980). The latter contained all the polymeric material i.e. DNA and poly(ADP-ribose). Each of the above fractions was concentrated by freeze-drying and analysed using thin layer chromatography (TLC). Figure 6.2 shows the results of a typical TLC analysis of the amino ethyl cellulose column fractionated material. As expected there is a significant amount of 3'AMP (curve ■———■). There is also a large amount of 5'AMP (curve ■———■). This is unambiguous evidence that some of the adenosine-pellet consists of mono ADPR/protein material. If the area under each curve is calculated, then the acetic acid fraction consists of 26% TLC origin material, 44% 5'AMP and 29% 3'AMP. The origin material in this TLC system is possibly compound X formed by base treatment of ADP-ribose. It is categorically not DNA and/or poly(ADP-ribose) because they do not elute in the 6M acetic acid wash of this column. If the ethylamine fraction is analysed on TLC then it can also be seen in Figure 6.2 (curve o———o) that over 90% of the counts are at the origin (note that the origin contains over 80,000 cpm). The ethylamine fraction was digested with snake venom phosphodiesterase. If the adenosine-pellet contained polymer, then snake venom phosphodiesterase (SVPDE) would produce 5'AMP and phosphoribosyl-AMP (PR-AMP). Spleen phosphodiesterase would have no effect on polymer. However if the fraction contained DNA (it cannot contain RNA because the base treatment has hydrolysed it all), snake venom

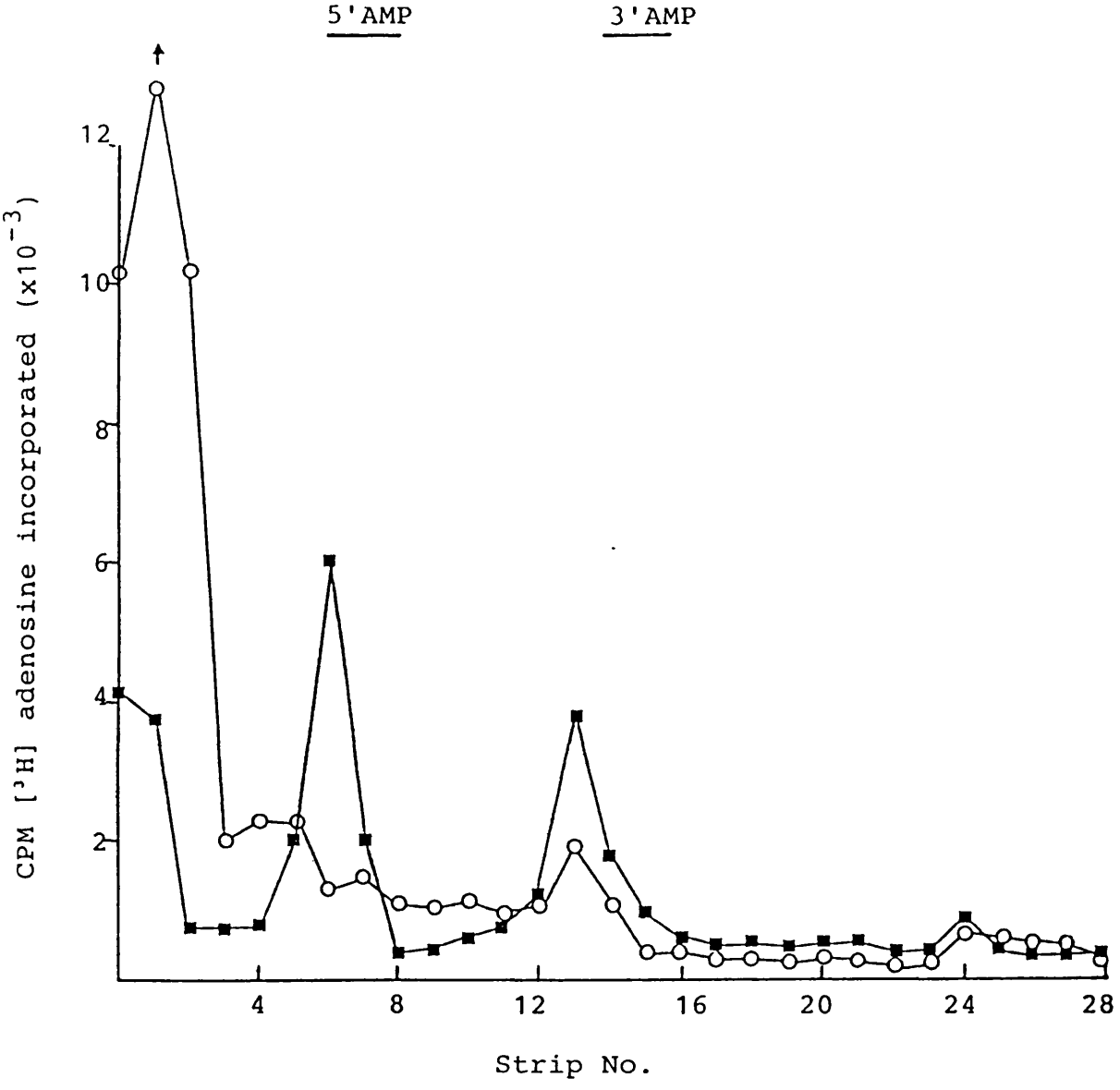
Figure 6.2.

Borate-complex T.L.C. of monomeric and polymeric material

Samples of the 6M acetic acid and 1M ethylamine fractions were freeze-dried and the residue dissolved in distilled water. The T.L.C. was carred out on poly-ethylineimine flexible plates (20cm x 20cm). These were developed in a single solvent system consisting of 0.15M sodium tetraborate/0.1M boric acid pH 7 in distilled water. The plate was run for 15cms. The radioactivity was determined by cutting the plate into 5cm pieces which were then individually counted (Section 3.2.10.3.).

O———O Is a plot of the distribution of radioactivity on the T.L.C. when ethylamine material was applied to the plate and run in the borate system.

■———■ Shows the distribution of radioactivity when the 6M acetic acid eluant (from above) is subjected to borate T.L.C.


5'AMP
3'AMP
12
10
8
6
4
2
CPM [³H] adenosine incorporated (x10⁻³)
4
8
12
16
20
24
28
Strip No.

phosphodiesterase will give 5'dAMP and the spleen enzyme will give 3'dAMP.

What is evident from Figure 6.3 is that the snake venom phosphodiesterase (SVPDE) completely hydrolyses the acid insoluble ethylamine fraction, i.e. there are no counts remaining on the origin. The second point is that the SVPDE does not give rise to any 5'AMP or PRAMP (the latter runs just ahead of the ADP-ribose marker). Not surprisingly there is a large peak at the position of 5'dAMP. Further analysis using alkaline phosphatase digestion of this material showed that it gave rise to 2 deoxy adenosine (curve now shown). This is, of course, the expected product from DNA digested with SVPDE plus alkaline phosphatase. The spleen diesterase digestion was also disappointing. This enzyme digested all the acid insoluble material to acid soluble products (i.e. no cpm remaining on origin) (see Figure 6.4) which is unambiguous evidence that none, or very little, of the adenosine label in the ethylamine fraction is present as poly(ADP-ribose). Experience in this laboratory has shown that in the borate TLC system dimeric ADP-ribose will not move off the origin, whereas monomer does. It is difficult to interpret the remaining peaks on the chromatogram. The "ADPR like" peak control cannot in fact be ADP-ribose because the latter is 100% destroyed by the initial base treatment. The 5'dAMP arises from DNA. The picture described does not change even if embryos are germinated in the presence of 500μCi/millilitre [^{3}H]

Figure 6.3.

Thin layer chromatography of "ethylamine fraction" which had been exhaustively hydrolysed by snake venom phosphodiesterase

Ethylamine fraction (see Figure 6.2 and page 111-112) was made up in 4M urea containing glucose-6-P (the latter acts as alternative substrate for non specific mono-esterases). After digestion 50µl of this material was applied to a PEI-plate (Section 3.2.9.) and developed in the borate solvent system for 15 cms. (See Figure 6.2).

■——■ The rate obtained when the above material was analysed on borate, PEI-cellulose T.L.C. 20 nmole of markers (ADPR, 5'-AMP, 3'AMP, adenosine) were always applied to the plate prior to the sample.

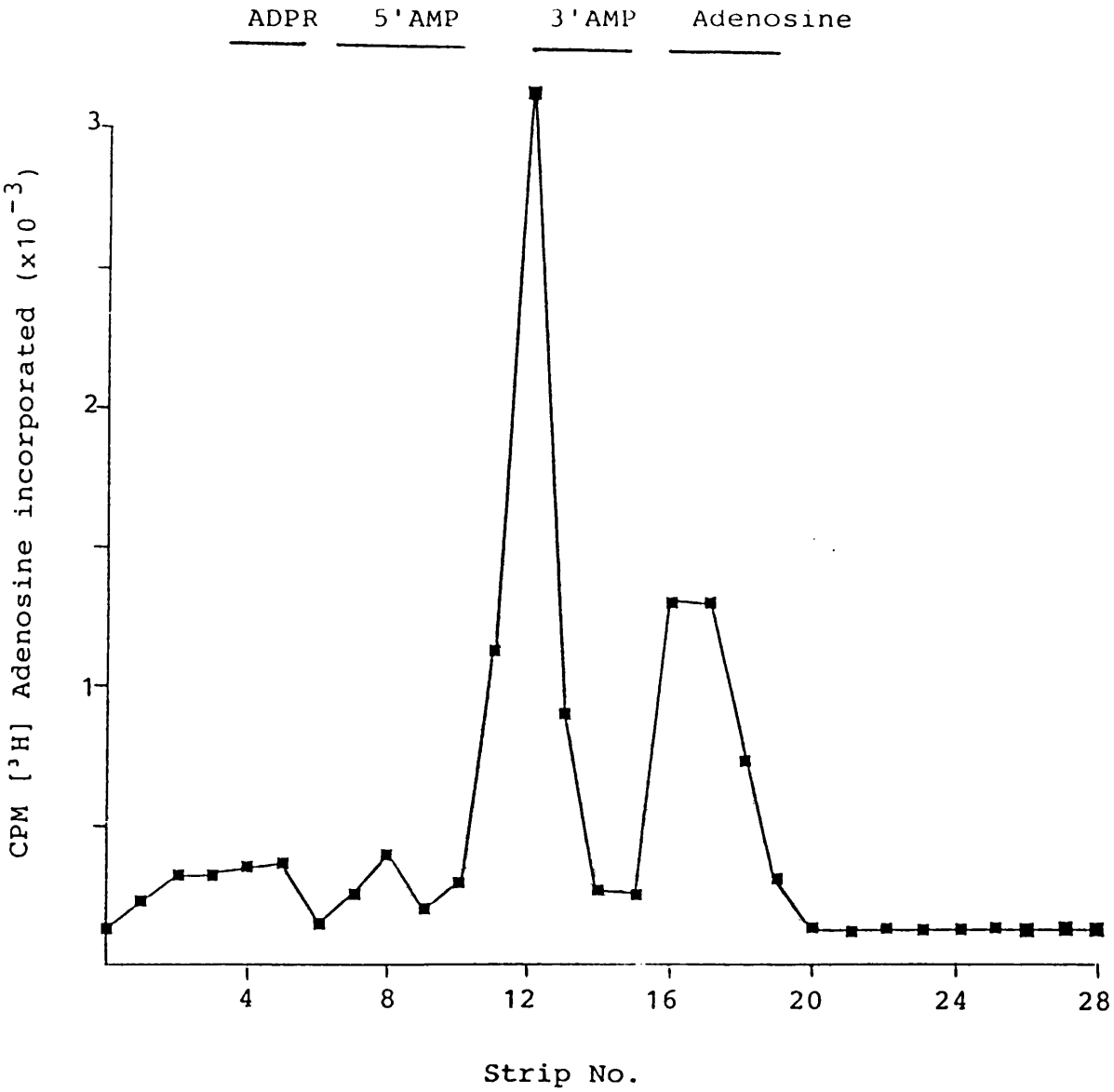
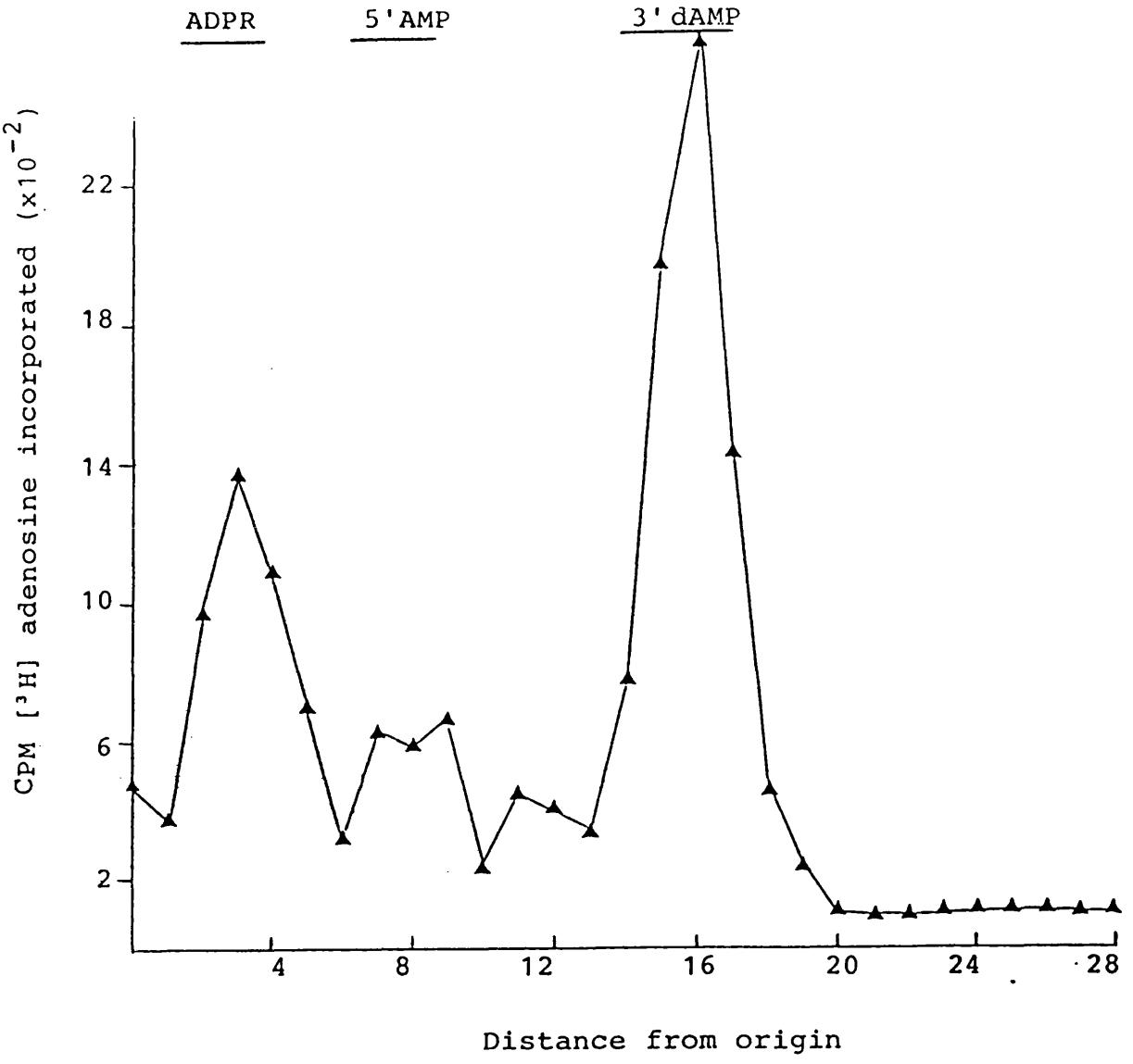

ADPR
5'AMP
3'AMP
Adenosine
CPM [³H] Adenosine incorporated (x10⁻³)
3
2
1
4
8
12
16
20
24
28
Strip No.

Figure 6.4.

T.L.C. analysis of spleen phosphodiesterase digested ethylamine material

Ethylamine material (Figure 6.2 and Section 6.1, page 111-112) was digested with spleen phosphodiesterase under the same conditions as described for snake venom phosphodiesterase (Figure 6.3 and Section 6.1, pages 109 and 110). The T.L.C. system is identical to that described in Figure 6.2.

20 nmoles of ADPR, 5'-AMP, 3-dAMP were run as U.V. markers.


ADPR
5'AMP
3'dAMP
CPM [³H] adenosine incorporated (x10⁻²)
22
18
14
10
6
2
4
8
12
16
20
24
28
Distance from origin

adenosine. Even at this very high level of isotope, no significant amount of polymer can be detected. One must conclude therefore that the level of poly(ADP-ribose) in germinating wheat embryos is below the level which is detectable by current methods. However, it is exciting that base treatment of the acid insoluble [^{3}H]adenosine pellet does give rise to 5'AMP which under these conditions can only arise from ADP-ribose bound to proteins.

Note has to be taken of the fact that when using [^{3}H]adenosine labelling in wheat embryos, the label appears in DNA which seems to behave in an anomalous fashion on the gradients. In other words, the caesium chloride urea gradient behaves as expected with [^{3}H]thymidine as precursor, and no [^{3}H]thymidine is found on the top of the gradient. But when [^{3}H]adenosine label is used, a caesium chloride buoyant DNA species is found which co-bands with proteins and not with normal DNA or RNA.

It is possible that the wheat embryo contains a DNA fragment which is bound to proteins. It is difficult however to see why this DNA would not be thymidine labelled in the [^{3}H]thymidine experiments (see conclusion).

It must be stressed that the data presented here comes from one complete experiment. The gradient was repeated 23 times using a variety of ^{3}H-adenosine concentrations and the picture described in this Section was seen for each analysis. Though no polymer could be found, it was felt that there was

a sufficient mono ADP-ribosylated protein present in the "adenosine pellet" to warrant further experiments directed towards finding which proteins might be modified. The most obvious candidates are the histones and the experiments concerning them are outlined in the next chapter.

CHAPTER 7

Determination of Putative ADP-Ribose Complexes

The histones are a group of very easily purified proteins which are also well characterised in the literature (see Section 2.3). They are soluble in mineral acid and insoluble in 20% TCA. These solubility properties in acid form the basis of all published purification methods. A further advantage with the methodology is that it is amenable for use with the "adenosine-pellet" material used in the last Chapter, as well as with whole cells, embryos or tissue. This means that the ADP-ribosylated histones may be isolated directly from wheat embryos germinated in ^{3}H-adenosine.

The first and simplest approach therefore, was to germinate embryos as described in Section 3.2.1. in the presence of 50 µCi/ml adenosine. The embryos were left in the dark at 26°C for 6 days. This long period of incubation was used so that the maximum possible amount of radiolabelled protein would be formed. The embryos were harvested and washed well in germinating medium. They were

then homogenised in 0.1M H_2SO_4 to acid precipitate all DNA, RNA and most proteins except for the histones (see Materials and Methods Section3.2.5). The method only differed in that the final freeze-dried histones were dissolved in distilled water and left for one hour with occasional shaking. After this time, the insoluble material (the amount was usually small) was removed by low speed centrifugation in a bench centrifuge. The supernatant was used for all subsequent experiments. The reproducibility of this isolation procedure varied considerably between various identical germination experiments. However the acid insoluble adenosine associated with this histone material rarely dropped below a total of 25,000 cpm 20%TCA insoluble. A 50µl aliquot of histone proteins was then run on SDS polyacrylamide gel electrophoresis which was stained, destained and scanned on a Pye-Unicam SP1800 gel scanner at 620nM (see Materials and Methods Section 3.2.11).

Figure 7.1 (solid line) shows a typical scan. The positions of highly purified histone markers are indicated at the top of the diagram. A second gel which had been electrophoresed in an identical manner was cut into 1.5mm slices and the radioisotope determined in a scintillation liquid by the method of Aloyo (see Materials and Methods Section 3.2.11). Figure 7.1 also shows the radioactivity content of each slide (broken line).

It is quite apparent that though the preparation contains all the expected histones, not one of these was

Figure 7.1.

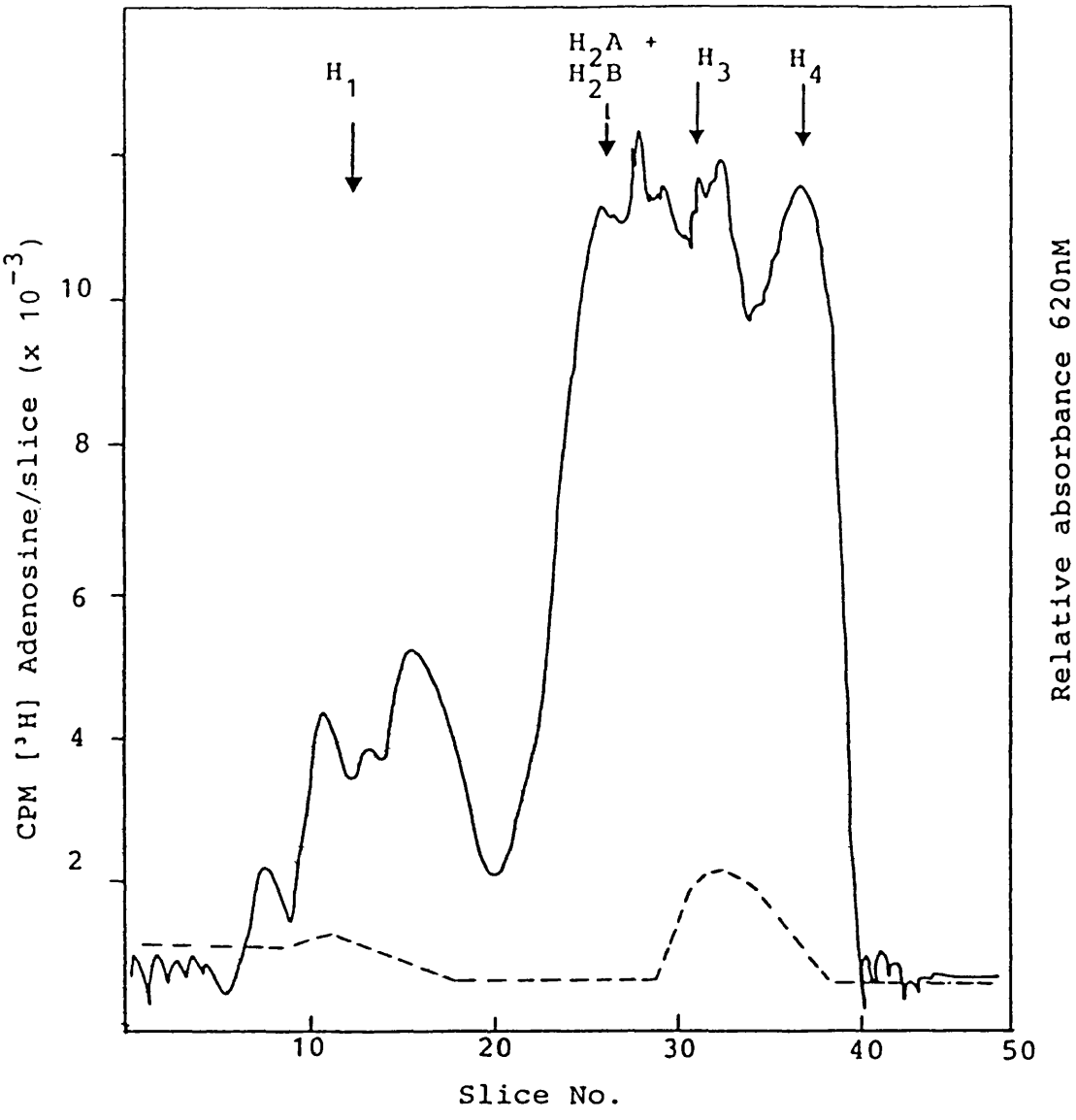
Distribution of radiolabelled protein from 6 day old germinating wheat embryos.
SDS-Polyacrylamide gels

Histones were isolated from embryos exactly as described in Section 3.2.5. page 73. SDS-polyacrylamide disk gels were prepared as outlined in Section 3.2.11.4. page. 81. 50µl of the histone preparation was run on each gel. When the electrophoretic run was finished, the gels were stained and destained (Section 3.2.11.3.) and then scanned using a Pye-Unicam SP1800 scanner.

The markers indicate where purified histones ran.

Radioactivity was determined when the gels had been sliced into 1.5mm pieces and swollen by the method of Aloyo (Section 3.2.11.2).

The dotted line indicates radioactivity. The solid line represents the Coomassie blue stained material.


H1
H2A + H2B
H3
H4
CPM [3H] Adenosine/slice (x 10-3)
Relative absorbance 620nM
Slice No.
2
4
6
8
10
10
20
30
40
50

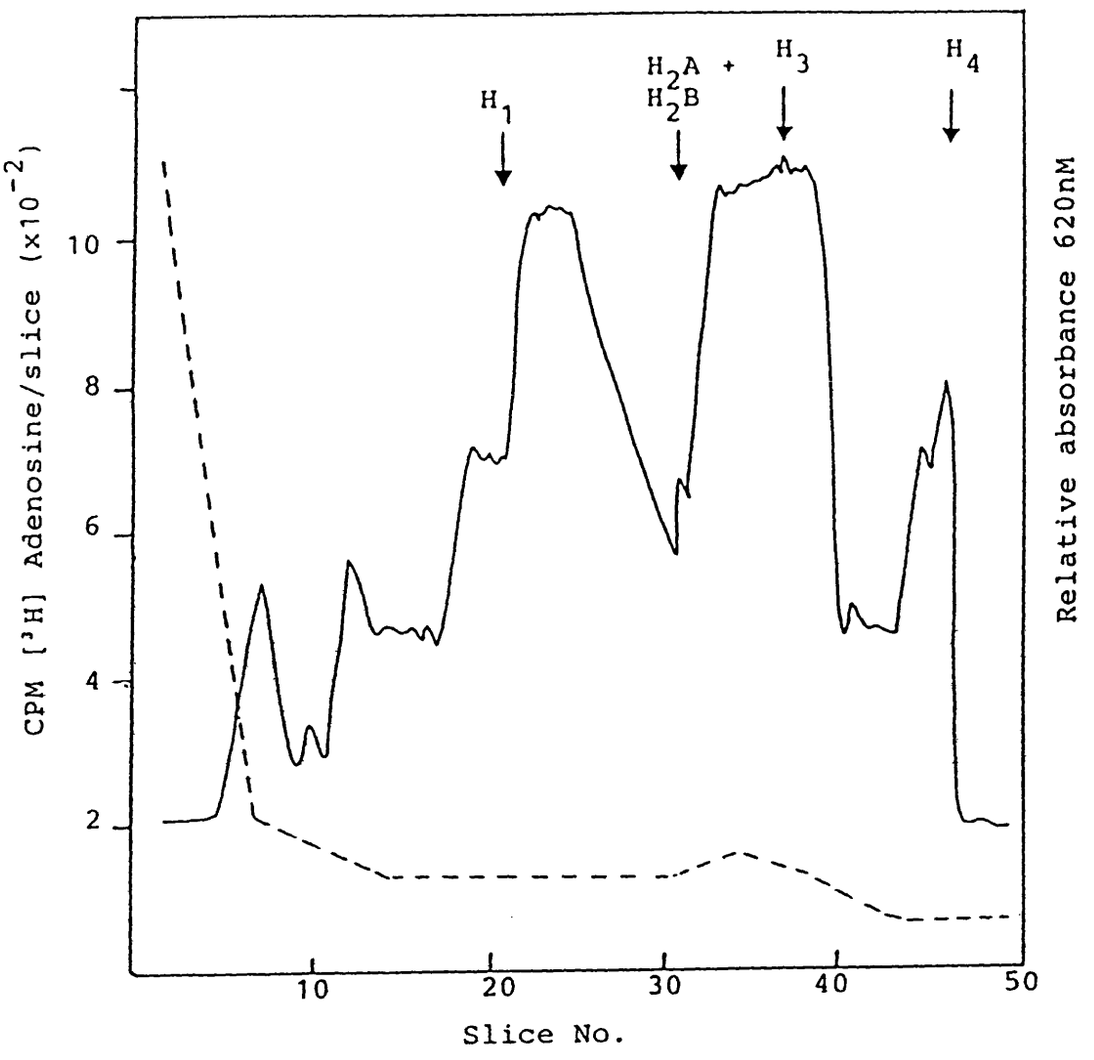
obviously labelled with [^{3}H]adenosine. It is possible that the long labelling time used during the germination period may have allowed cellular breakdown of [^{3}H]adenosine into a variety of metabolites none of which are involved with histones. The problem here is that there is no literature available on purine/pyrimidine interconversions and metabolism in seeds or seed embryos. To minimise this possibility the labelling time was reduced to 72 hours and the histone extraction and electrophoresis repeated. An SDS polyacrylamide gel was essentially the same as shown already in Figure 7.1. The histones were run in a second electrophoresis system which might have improved the resolution. Figure 7.2 shows both the coomassie blue (solid line) and the radioactive (broken line) profiles. The histone separation is, as expected, somewhat better on acid urea gels than on the SDS system. However a number of problems are evident when analysing the radioactive profiles. The first is that no histone is obviously labelled. It is possible that H_3 contains some isotope, but the others definitely do not. The second major problem - a relatively common one in non-SDS gels - is that most of the radioactivity does not enter the gel. This is likely to be due to aggregation of the proteins even though there is 6M urea present during electrophoresis.

The ^{3}H-labelled slices were removed from the scintillant and analysed for 5'AMP content after base treatment. None could be detected. It must be stressed that this can only be

Figure 7.2.

Histone extraction from 72 hour germinating wheat embryos. Acetic acid-urea gels

The methodology was identical to that described in Section 3.2.5. and Figure 7.1, except that histone material was isolated from 72 hour incubated embryos.


CPM [³H] Adenosine/slice (x10⁻²)
Relative absorbance 620nM
H1
H2A + H2B
H3
H4
2
4
6
8
10
10
20
30
40
50
Slice No.

an indication that no 5'AMP is present, because the counts were very low and thus difficult to quantitate. Furthermore the unique peak seen in the isotope profile of Figure 7.2 was not a reproducible one. The overall experiment outlined above was repeated with embryos germinated in [^{3}H] adenosine for 24 hours. The acid-urea electrophoresis system described for Figure 7.2 was used. As seen in both previous figures, the histones are very clearly separated but none of them is adenosine labelled. There is a hint that some isotope migrates with H_1 and H_4. The problem here is that though the coomassie blue scans are very reproducible, the isotope scans are not. The major problem, already mentioned above, is that much of the labelled material does not enter the gels. Thus the reliability of these experiments is suspect.

One possibility which might explain the lack of labelled histone in the gels is that during the isolation procedure, some aggregation of the labelled histones might have occurred. Figure 7.3 shows that this is unlikely because the histones are labelled and all the label has entered the gel. It is obvious from Figure 7.3 that only very fast migrating labelled material is present in the supernatant and that this migrates faster than all the histones. Though this was not analysed, the reproducibly fast migration seen in every experiment with the histone supernatant rules out the possibility that this is an ADP-ribosylated protein .

Figure 7.3.

Acetic acid-urea polyacrylamide gel of the histone supernatant

Histones were isolated from 24 hour germinated wheat embryos (Section 3.2.5., page 73). 50µl of histone supernatant (page 118) was applied to disc gels and run as described in Section 3.2.11.3. page 80 and Figure 7.1.

The solid line represents the Coomassie blue staining material. The dotted line indicates the radioactive content of each gel slice.

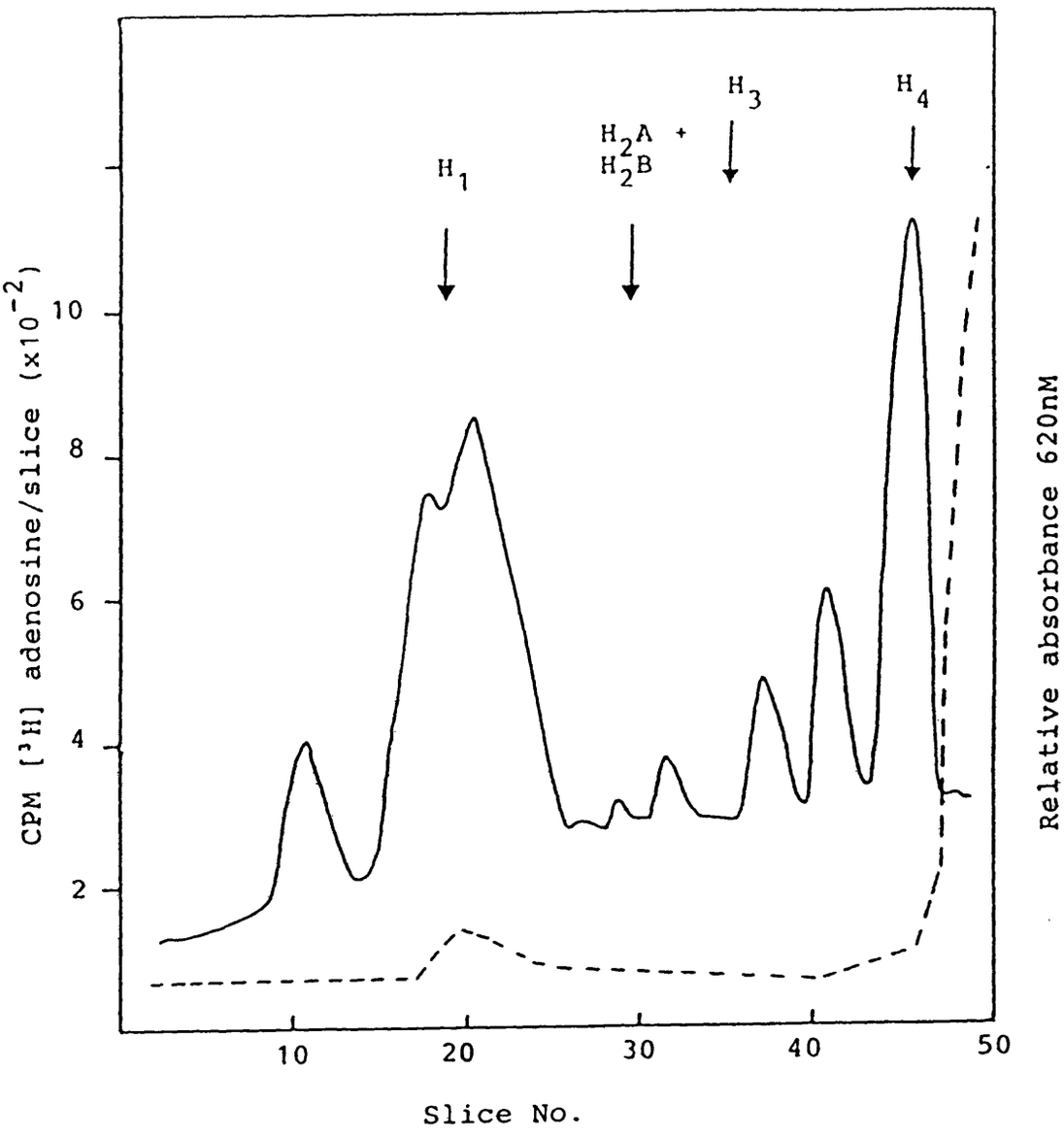

H1
H2A + H2B
H3
H4
CPM [3H] adenosine/slice (x10-2)
Relative absorbance 620nM
Slice No.
2
4
6
8
10
10
20
30
40
50

Figure 7.4.

Acid-urea gel electrophoresis of proteins radiolabelled *in vivo* during different periods of germination in [^{3}H]adenosine

Embryos were incubated with [^{3}H]adenosine for different periods of time. Subsequently the histones were isolated (Section 3.2.5.) and run on polyacrylamide gels (Section 3.2.11.3. and Figure 7.1.).

o——o embryos germinated for 2 hours in [^{3}H]adenosine

●——● embryos germinated for 4 hours in [^{3}H]adenosine

□——□ embryos germinated for 8 hours in [^{3}H]adenosine

N.B. These gels were not stained and scanned, but sliced and the radiolabel counted in each slice (Section 3.2.11.2.).

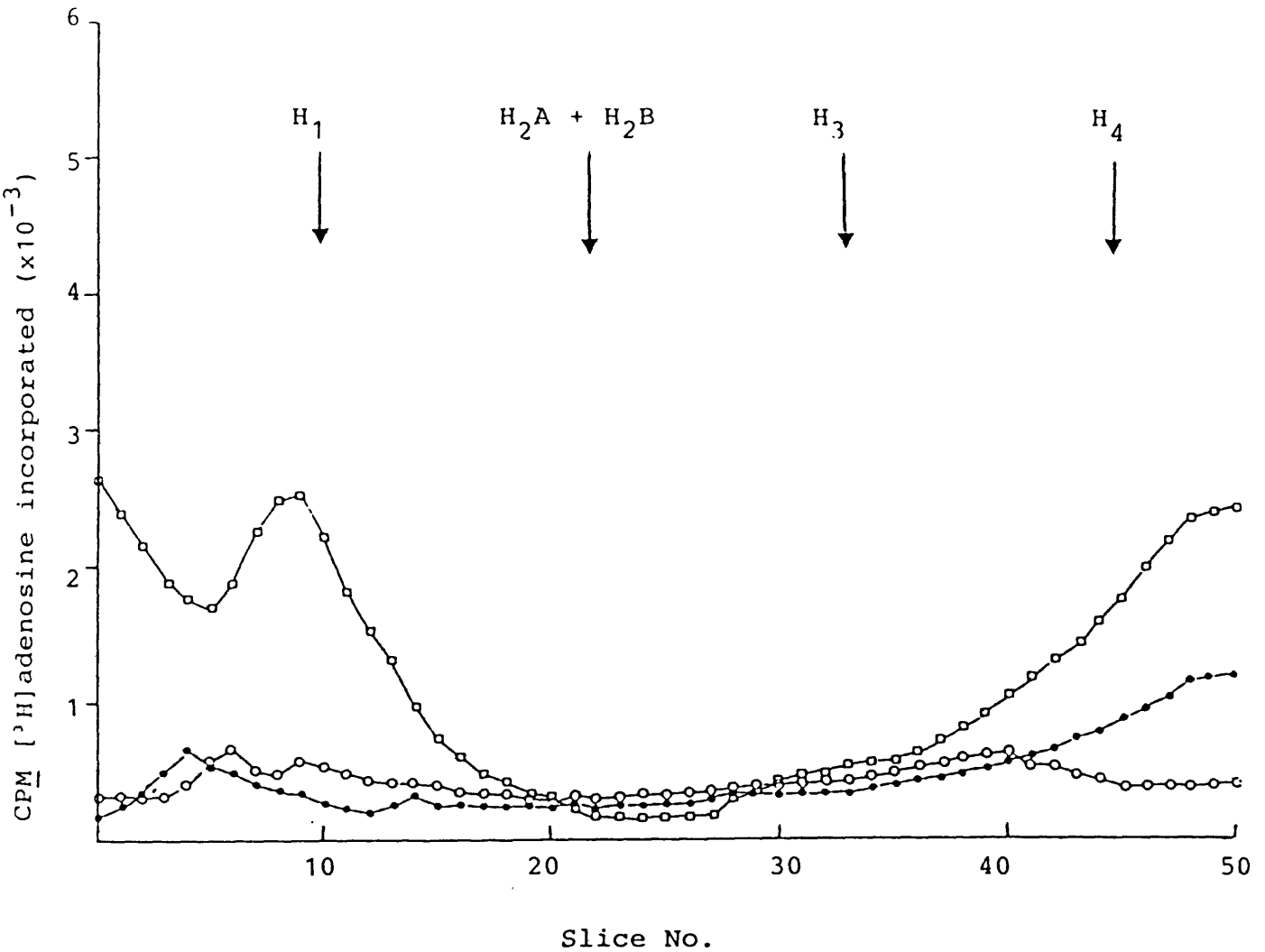

6
5
4
3
2
1
H_1
H_2A + H_2B
H_3
H_4
CPM [^{3}H]adenosine incorporated ($\times 10^{-3}$)
10
20
30
40
50
Slice No.

Figure 7.5.

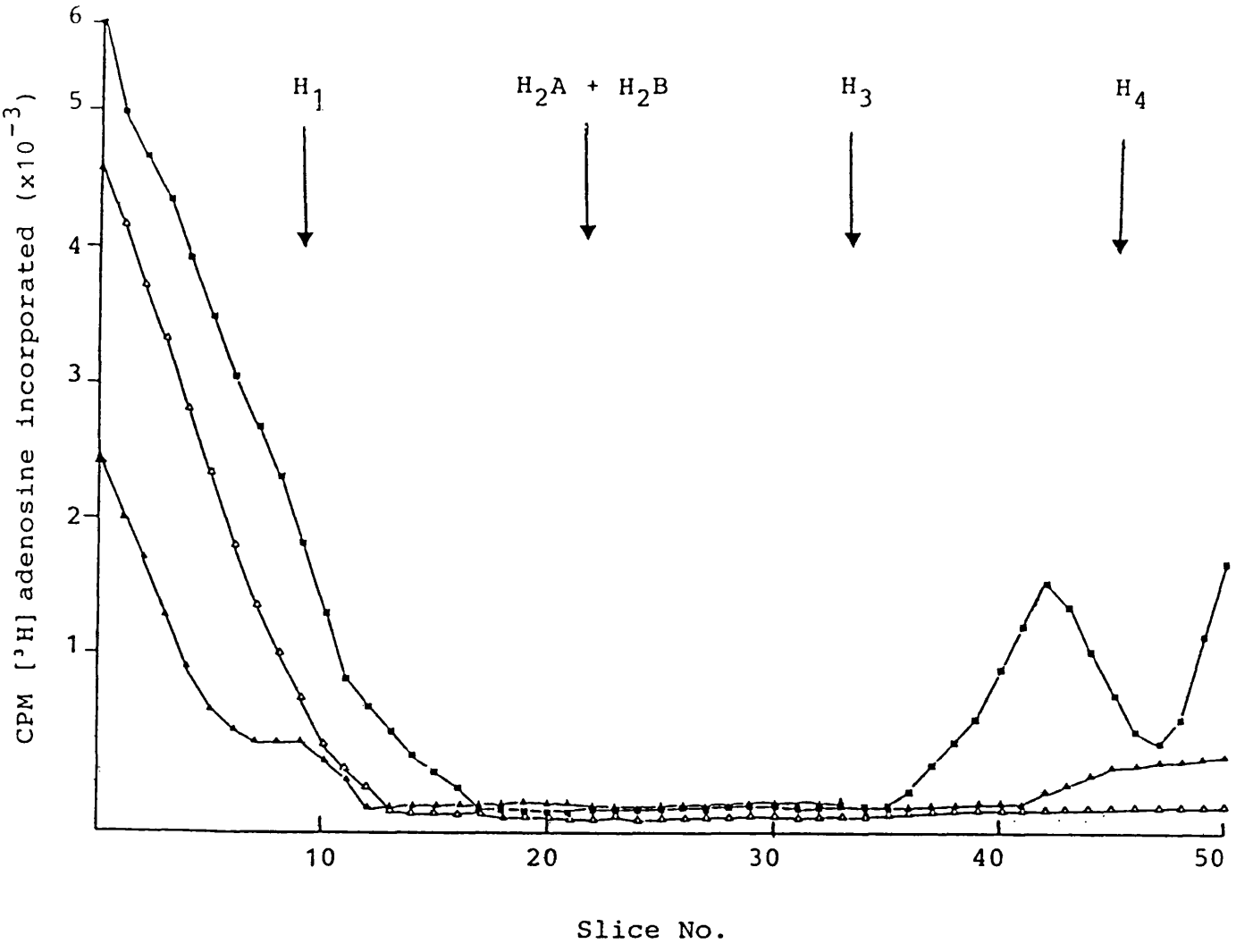
Acid-urea gel electrophoresis of proteins radiolabelled *in vivo* during different periods of germination in [^{3}H]adenosine

Embryos were incubated with [^{3}H]adenosine as outlined in Figure 7.4. Histones were isolated and run on polyacrylamide gels and the radioactive profile of each gel determined (Figure 7.4.).

△——△ 16 hours germination in [^{3}H]adenosine

■——■ 20 hours germination in [^{3}H]adenosine

▲——▲ 24 hours germination in [^{3}H]adenosine


6
5
4
3
2
1
CPM [³H] adenosine incorporated (x10⁻³)
H1
H2A + H2B
H3
H4
10
20
30
40
50
Slice No.

The experiments outlined earlier where embryos were labelled for 72 or 24 hours with [^{3}H]adenosine were repeated but the labelling time was varied between 2 hours and 24 hours (Figures 7.4 and 7.5).

The curves obtained are interesting for a number of reasons. Even at 2 hours, some traces of label run into the gel (curve o——o). At 4 hours all the isotope enters the gels but it appears to be small fast migrating material (curve ●——●). The picture changes quite dramatically at 8 hours germination (curve □——□). The amount of label not entering the gels is high and there is a broad peak of labelled protein at fraction 10. This does not correspond to any of the histones. There is also a broad band at the bottom of the gel starting at fraction 40, onwards. This peak coincides with H_4. As the germination time in [^{3}H]adenosine increases, the labelling pattern changes quite markedly. In Figure 7.5, a large amount of the total radioactivity is at the top of the gel. The amount of this slow moving species varies greatly and does not appear to be directly related to germination time. At 20 hours (curve ■——■) there is a peak of isotope starting at fraction 40. However this is missing at 16 hours and 24 hours. It may be that there is a specific labelling of H_4 at defined times during germination (8 hours and 20 hours) (see conclusion). The gel patterns may represent labelled proteins which are contaminants in the predominatly (90%) histone protein preparation.

It is clear that the only histone which might be labelled is H_4. It is equally clear that the other histones are not labelled. The latter finding must be viewed with caution because there may not be sufficient high specific activity NAD formed to allow synthesis of detectably radioactive ADP-ribosylated histones. The 24 hours germination was repeated at 1mCi [^{3}H]adenosine per millilitre of germination medium. Gel electrophoresis of the histones from this very high specific radioactivity germination failed to show any labelled histones. There was a small peak of activity at the H_4 position but this peak was not enlarged in radioactive content compared to the previous experiments at 50μCi/ml [^{3}H] adenosine (data not shown).

It is clear that if the histones are modified with ADP-ribose, there is none detectable by the methods described above. It is also apparent that the mono ADP-ribosylated proteins are not histones.

When this work was started, the use of [^{3}H]adenosine as a precursor for ADP-ribosylation was the only approach available. It is unsatifactory because absence of [^{3}H] adenosine in the histones may simply mean that the specific radioactivity of the cellular [^{3}H]NAD is too low to detect. An attempt was made to determine histone ADP-ribosylation using a chemical method.

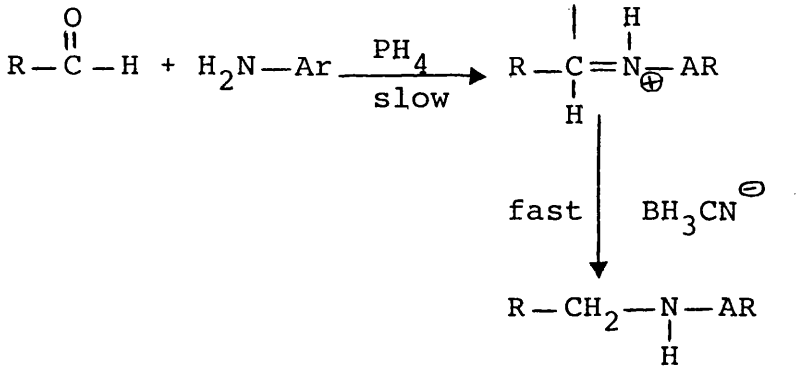
CHAPTER 8

8.1. Chemical Analysis of Histone ADP-Ribosylation

Quantitation of protein ADP-ribosylation *in vivo* is not yet possible. Whilst many methods have been published, none of them has turned out to be of general use.

The histones are known not to be glycosylated proteins. The chemical analysis now described exploits this property as follows. Many sugars (including ribose) contain a cis-diol grouping of their hydroxyl groups. These cis-diols are susceptible to oxidation with sodium periodate which produces a dialdehyde (Parish, 1972 and Whitfeld, 1954). The dialdehyde once formed can be reacted with a primary aromatic amine(4-amino thiophenol) to form a double Schiffs base (imine) (see diagram below). The reactions outlined here have been used in a variety of ways in the early days of RNA sequencing (Zamecnik, P. 1960., Steinschneider & Fraenkel-Conrat, 1966., Neu & Heppel, 1964, Khym & Cohn, 1961, Leppla *et al*., 1968). The imine so formed can be stabilised by reduction with sodium cyanoborohydride (Na $CNBH_3$). This produces the corresponding secondary amine. The advantage of using cyanoborohydride for the reduction is that while the imine is reduced the unreacted aldehyde is not. Hence this reductive amination can be carried out in one step,

i.e. the cis-diol is first oxidised, excess sodium periodate is removed, the aromatic amine and cyanborohydride are mixed and amination will proceed (see diagram below).



Ar = aromatic

The conditions for this reaction can be as low as pH 3.0. This is possible because the CN-group in the cyanoborohydride deactivates the borohydride ion and renders it stable to low pH's. Under similar conditions sodium borohydride is explosively unstable.

The idea, therefore, is to take unradiolabelled pure histones which will contain only ADP-ribose and no other glycohysidic, cis-diol containing sugars.

The histones may then be oxidised with periodate, acid precipitated and washed to remove excess periodate, and reacted with an aromatic amine and cyanoborohydride. The complex so formed (in this case the secondary amine), may be detected in a number of ways:

(a) the added aromatic amine may be radioactive;

(b) the cyanoborohydride may be tritiated $CNBH_2T$

(c) both amine and cyanoborohydride are radioactive

(d) the aromatic amine might carry an easily characterisable group.

The method chosen was (d). The reasons for this are that radiolabelled aniline (a) is available but its volatility and instability in air (to form quinones) and not least, its expense, make aniline difficult to use. Tritiated sodium cyanoborohydride (b) is available commercially but a serious drawback is that its specific radioactivity is extremely difficult to determine. Thus the method of choice is to use an aromatic amine with an easily characterisable functional group. The best candidate seemed to be 4-amino thio-phenol. Use of this molecule gives quite a dramatic and initially an unseen, advantage. Each molecule of ADP-ribose contains two ribose moities which on oxidation give 4 aldehyde groups. Reaction of these with 4-amino thio phenol and sodium cyanoborohydride will incorporate 4 thiol groups per molecule of ADP-ribose. $(\text{ADP-ribose})_n$ is somewhat more complex because whereas the adenine ribose contains a cis-diol, the other ribose (to which the nicotinamide was originally attached) is linked via a 1'-2' link to its neighbouring ADP-ribose. This 1'-2' link thus effectively removes one cis-diol.

The initial plan was to detect these using standard SH reagents such as radioactive bis-dithionitrobenzoic acid (DTNB) or 4-4' pyridyl disulphide. These compounds

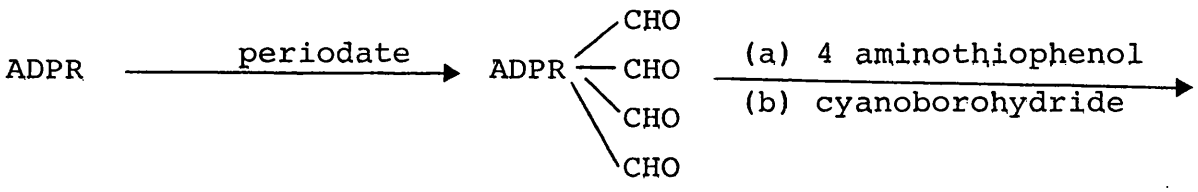
are very expensive in their radioactive form. A search was made for a more sensitive SH reagent and it was found that mercury-203 (^{203}Hg) modifies SH groups quantitatively. Furthermore ^{203}Hg is a powerful γ emitter and is therefore easily detected in a gamma counter with great efficiency but with none of the quenching problems usually seen with tritium. However its drawback is that mercury forms bivalent links with thiols e.g.

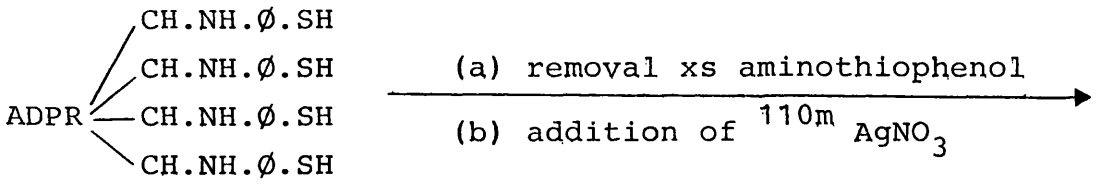
R — S — Hg — S-R.

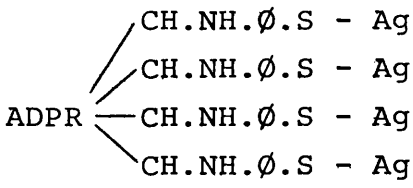
The obvious disadvantage therefore is that this might cause crosslinking and insolubility problems with ADP-ribose proteins, i.e. large very high molecular weight aggregates may be found.

Thiols react with many heavy metals. A consideration of all these resulted in the use of radioactive silver (^{110m}Ag). Like ^{203}Hg, ^{110m}Ag is a powerful gamma emitter. Unlike ^{203}Hg however, ^{110m}Ag (or non radioactive silver) reacts with thiols in a monovalent fashion to form silver mercaptides which cannot crosslink.

The chemistry of this ADP-ribose detection method is briefly summarised in the scheme below.







Thus each ADP-ribose residue contains four radioactive silver atoms. It is interesting to note that even small amounts of very long polymer will be detectable because for example $(ADPR)_{20}$ will contain 42 atoms of ^{110m}Ag.

A serious drawback to the above theoretical methodology is that pure poly(ADP-ribose) of known chain length is not available so all the development work was done using adenosine 5'-monophosphate. The main reasons for this choice were that 5'-AMP is stable, it is available in a very pure form, it contains a cis-diol group and it was also available in the laboratory as $2^{3}H$-5'AMP.

8.2. Developmental Procedures for Silver Radiolabelling of Adenosine-5'-Monophosphate

The first step was to show that 5'AMP is quantitatively oxidised by sodium periodate at pH 6.0. T.L.C. on PEI

cellulose plates (Section 3.2.9.) was found to be the most simple and effective method of separation 5'AMP and its oxidised form. When these compounds were applied to a PEI plate and developed in 0.2M LiCl in water, the RF's of 5'AMP and oxidised 5'AMP were 0.50 and 0.10 respectively.

Next, 5'AMP (10 μmoles) was dissolved in 1 millilitre of 0.1M sodium citrate buffer pH 4.0. To this solution was added solid sodium periodate (20 μmole) which dissolved rapidly. The resulting solution was left on ice for 60 minutes. About 20 microlitre of this reaction mixture was applied to a PEI-cellulose plate and developed in 0.2M LiCl. When observed under ultraviolet light all the ultraviolet (U.V.) absorbing material had an RF of 0.1. There was no detectable 5'AMP. The experiment was repeated in almost exactly the same way except that 10^6 CPM of 2^3H-AMP was also added to the reaction mixture. Once again complete oxidation of the 5'AMP had occurred because all the counts had an RF of 0.1 (5'AMP dialdehyde). There were no detectable counts on the 5'AMP position. Thus the oxidation of 5'AMP was quantitative. The next step was to show quantitative Schiffs base formation when the oxidised 5'AMP was reductively aminated with 4-aminothiolphenol.

Once again the oxidation was a repeat of the above radioactive experiment. After oxidation had occurred the whole reaction mixture was poured over a Dowex-1-acetate anion exchange resin column (2cm longx1cm diameter).

The oxidised 5'AMP binds tightly to this column and the periodate only weakly. Thus periodate was removed by eluting the column with 10 millilitres 0.03M acetic acid. The oxidised 5'AMP was quantitatively eluted with 10 millilitres 1.0M acetic acid. This solution was freeze-dried. The dry oxidised 5'AMP (10 μmoles and 10^6CPM) was then dissolved in 1 millilitre 0.1M sodium citrate buffer pH 4.0 and to this was added 50 μmoles of 4-amino thio phenol (usually the latter was made up of a 0.5M solution in water and the appropriate aliquot added to the oxidised 5'AMP). Finally 50mgs of solid sodium cyanoborohydride was added to the above mixture, which was incubated at 37°C for 18 hours. This time of reduction may appear somewhat long but it was found to be necessary to obtain good results.

Not only does the cyanoborohydride reduce the Schiffs base as it forms, but it also prevents any disulphide formation from occurring. Moreover if the original 4-amino thio phenol contained the oxidised disulphide, this will be reduced to the free thiol.

After 18 hours incubation the reaction mixture was once again passed over a Dowex-1-acetate ion exchange column as described earlier. Excess 4-amino thiophenol was removed with 20 millilitres of distilled water. The bis(4-amino thiophenol)-5'AMP adduct was eluted with 1M acetic acid. After freeze drying the yield of radiolabel

was 90%. This is assumed to be the recovery yield of oxidised 5'AMP. Whether or not it contains 2 moles of 4-amino thio phenol per mole of oxidised 5'AMP is unknown at this stage.

A classical method for the determination of -SH groups is the use of 5,5'dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellmans reagent). This reagent reacts with thiols in aqueous media at pH 8.0. The released 5-thio nitrobenzoic acid has an extinction coefficient of 13600 at 412nm. Anderson and Wetlaufer (1975) have reported a simple, sensitive and reliable technique for assaying thiols with DTNB.

The bis (4-aminothiophenol)-oxidised 5'AMP adduct was dissolved in 10 millilitres of distilled water and then assayed using the method of Anderson and Wetlaufer without modification. Briefly their method is to treat the sample with strong NaOH to cleave disulphides (if any). The strong base is neutralised with phosphoric acid and the thiols are estimated colourimetrically with DTNB in the presence of EDTA (EDTA prevents heavy metal binding to thiols) at 412nm. Using this assay it was found that there were 1.96 moles (± 0.2) of thiol per mole of oxidised 5'AMP. This experiment was repeated ten times and the result was consistently 1.96 moles thiol per mole of oxidised 5'AMP. This is slightly under the 2:1 stoichiometry expected. It is likely this is because the reductive amination is not absolutely quantitative.

The final step is to show that silver binds to the bis(4-amino thio phenol)-oxidised 5'AMP adduct in the expected ratio. This was done in two ways. First, the above adduct was made up in 20% ethanol/water (v/v) at a concentration of 10µmoles in 1 millilitre (the radioactive content was normally used to determine concentration). An excess of silver nitrate was then added (50 µmole). After 30 minutes the reaction mixture was chromatographed on an aminoethyl cellulose column (see Materials and Methods, Section 3.2.8).

The silver ions do not stick to this mildly basic anion exchanger and they were removed with water. The silver-bis(4 amino thio phenol)-oxidised 5'AMP is eluted with 0.1M ammonium bicarbonate buffer, pH 8.3. The change of column strategy at this point is necessary because the silver-thiol complex is stable between pH 8-10 but is unstable below pH 4.0. At low pH values it was found that the silver dissociated from the thiol. The silver-thiol-oxidised AMP complex was not purified further. It was simply mixed with an ethanolic solution of DTNB. No colour at 412nm was produced. It can therefore be confidently calculated that all the thiol groups were convalently bound to silver and thus totally unavailable to reaction with DTNB.

The second way of showing that the silver reacted with the thiols was through the use of radioactive silver nitrate- $^{110m}Ag\ NO_3$.

The experiment was essentially the same as described earlier. However instead of adding an excess of unradioactive $AgNO_3$, an excess of known specific activity $^{110m}AgNO_3$ was added to the bis(4-amino thio phenol)-oxidised 5'AMP adduct. The silverated complex was purified and the specific radioactivity of the silver mercaptide of bis(4-amino thio phenol)-oxidised 5'AMP determined. An attempt was made to follow the time course of silveration of the thiols but the reaction was almost instantaneous. Thus, a 2 millilitre solution containing 5μmoles of bis(4-amino thio phenol)-oxidised 5'AMP in 20% ethanol was mixed with 20μmoles of $^{110m}AgNO_3$ (specific radioactivity 122500 cpm/μmole). The silver mercaptide of bis(4-amino silver thio phenol)-oxidised 5'AMP was purified, isolated and counted as before. It was found that there were 231,525 cpm ^{110m}Ag per 1 μmole of bis(4-amino thio phenol)-oxidised 5'AMP. The molar ratio of Ag:bis(4-amino thio phenol)-oxidised 5'AMP was therefore 1.89:1. Before attempting to do rigorous control experiments to ensure that this reductive amination and silver labelling method was quantitative for protein (ADP-ribose) it was decided to check the purified histones for groups which might react using this method. First, however, some cautionary comments need to be made.

1. The method as developed so far cannot be used if glycoproteins are present since these will be reductively aminated if the sugar residues contain cis-diols.

2. Before oxidation-reduction is carried out, free thiols present in the protein must be removed or blocked otherwise they will react with the silver in the final step. This is not a serious problem because a control can always be run where the protein fraction is reacted with silver without any oxidation or reductive amination. This value is then subtracted from the value obtained when the full reductive amination is carried out to give the radioisotopic content due to cis-diols.

3. It has been found in this laboratory (Purnell, unpublished result) that silver mercaptides are generally unstable in strong acids. The silver is removed from the thiol group. Thus 20% TCA precipitation cannot be used to determine the protein-silver mercaptide content. The quickest and most reliable method is to use gel filtration (see Materials and Methods Section 3.2.11).

Histones were purified as previously described (section 3.2.5).The freeze-dried histone preparation was dissolved in 0.1M sodium citrate pH 4.0 to a concentration of 1mg per millilitre of buffer. To ten millilitres of this solution was added 100mgs solid sodium periodate. This reaction mixture was mixed well and left on ice for two hours. At this time, ice-cold 100% TCA (w/v) was added to a final concentration of 25% TCA (w/v). This was left on ice for two hours and then centrifuged at 40,000xg in an MSE 18 centrifuge. The pellet was collected

and sonicated in 50 millilitres 25% ice cold TCA (w/v) and the resulting finely dispersed suspension centrifuged as before. The supernatant was again discarded and the pellet resuspended in 25% ice-cold TCA by sonication. After centrifugation the pellet was rewashed by this procedure 5x to remove all the sodium periodate. The pellet was resuspended in ice cold 80% acetone (in water v/v) and centrifuged again. This last step removed most of the TCA.

The acetone washed pellet was taken up in five millilitres 0.2M sodium citrate buffer pH 4.0. To this suspension was added 20μmoles of 4-amino thio phenol and 1000 μmoles sodium cyanoborohydride (36mgs). These quantities are likely to be far in excess of the dialdehyde concentration in the oxidised histones. The above reaction mixture was left at 37°C for 18 hours and the suspension became a solution. After this time the histones were once again acid precipitated and washed 5x in 25% ice cold TCA as described earlier. If thiol groups have been introduced into the histones by this process, the acid washing has to be exhaustive and complete. The main advantage of using these very acidic conditions at this stage is that thiols oxidise only very slow to disulphides (a $t_{\frac{1}{2}}$ of weeks) at acid pH's (Pharmacia, 1977). The acid pellet was washed in acetone as described earlier, but at this stage it was carried out under a

stream of nitrogen (to protect the thiols). Finally the pellet was taken up in five millilitres 1.0M sodium acetate buffer pH 6.5 and an excess of $^{110m}AgNO_3$ was added (10^6cpm per nanomole of $^{110m}Ag^+$) and the mixture stirred for 2 hours at room temperature. After this time the reaction mixture was applied to a Biogel P4 gel column (2.5cm x 50cm) which has been equilibrated in 1.0M sodium acetate pH 6.0. The high acetate concentration was used to minimize ionic interactions between protein carboxyl groups and $^{110m}Ag^+$. The radioactivity in each fraction was then determined. The gel column efficiently separated protein (estimated by A_{280}) and $^{110m}Ag^+$, however no radioactivity above background was found in the protein fractions. This experiment was repeated three times and no protein-silver complex could be detected. The sensitivity of the method can easily be worked out. Usually 10mgs of purified histones were used and so if the average molecular weight is 20,000 then approximately 0.5 micromoles of histones have been used in each of these experiments. If one assumes only one ADP-ribose bound per molecule of histone, then a total of 2.0 micromoles $^{110m}Ag^+$ should bind to the proteins, i.e. 2×10^9 cpm The method detects 1000cpm with reasonable accuracy and so 1.0 picomole (10^3 cpm) of ADP-ribose is detectable, i.e. the latter figure suggests that the method should detect about one mono ADP-ribosylated protein per million

unmodified histones.

It is interesting that no $^{110m}Ag^{+}$ appeared in the protein peak. No attempt was made to block protein SH groups before the oxidation step and so even if disulphides did form or were present initially the cyanoborohydride would have reduced them to thiols. To check this possibility 10mgs of purified histones were dissolved in five millilitres of 0.2M sodium acetate buffer pH 6.0 and 100mgs sodium cyanoborohydride were added and the whole left for 18 hours. The mixture was then made to 25% TCA by addition of 100% TCA (w/v) and left on ice for 6 hours in a fume-cupboard. This acid treatment quantitatively destroys the cyanoborohydride (great care was taken with this step because HCN is produced). Finally the pH of the TCA solution was adjusted to 8.5 using 1M NaOH. Addition of DTNB to the reduced histone preparation caused no absorption to occur at 412 nm, i.e. there were no thiols detectable in the histones.

The conclusion must be that the histones in wheat embryos are not ADP-ribosylated. Certainly two independent methods (adenosine labelling and reductive amination) showed that the histones did not contain any detectable ADP-ribose.

It is possible that the reductive amination does not work for protein bound ADP-ribose. It is difficult to see why this might be and so an experiment was done to

show that ADP-ribose-protein does react.

Previous work in this laboratory (Whish, unpublished results) showed that free ADP-ribose would bind to polylysine (average M.W. 100,000 daltons) covalently. The coupling is thought to be via the reducing sugar (or anomeric C1 to which the nicotinamide would have been attached in NAD) and the E-amino groups of lysine residues. This adduct was to be used for antibody work in the laboratory. It was not known how many ADP-ribose molecules were attached to each mole of polylysine because the polylysine was highly hetrogeneous in molecular weight.

The experiment to label the ADP-ribose-polylysine adduct was carried out exactly as described earlier for the histones. The $^{110}Ag^{+}$ specific radioactivity was also identical. When the final material was run on the Biogel P4 column large quantities of radioactivity eluted in the same place as the polylysine ADP-ribose. This is unambiguous proof that the method works on protein bound ADP-ribose.

It seems certain therefore that the wheat histones do not contain any detectable bound ADP-ribose.

Figure 8.1.

Biogel P4 gel chromatography of histone and silver 110M

Purified histone from wheat embryo (Section 3.2.5.) (5mg) was dissolved in 2ml 1M sodium acetate pH 6.5. The column (50cm long x 0.9cm) was eluted with 1M sodium acetate pH 6.5. Each fraction was read at 280nm.

Radioactive silver nitrate ($^{110M}AgNo_3$) 50µl and 50,000 CPM was dissolved in 2ml 1M sodium acetate pH 6.5. This was applied to the Biogel P4 column (50cm long x 0.9cm). 200µl of each fraction was counted in an LKB-Walloch gamma counter.

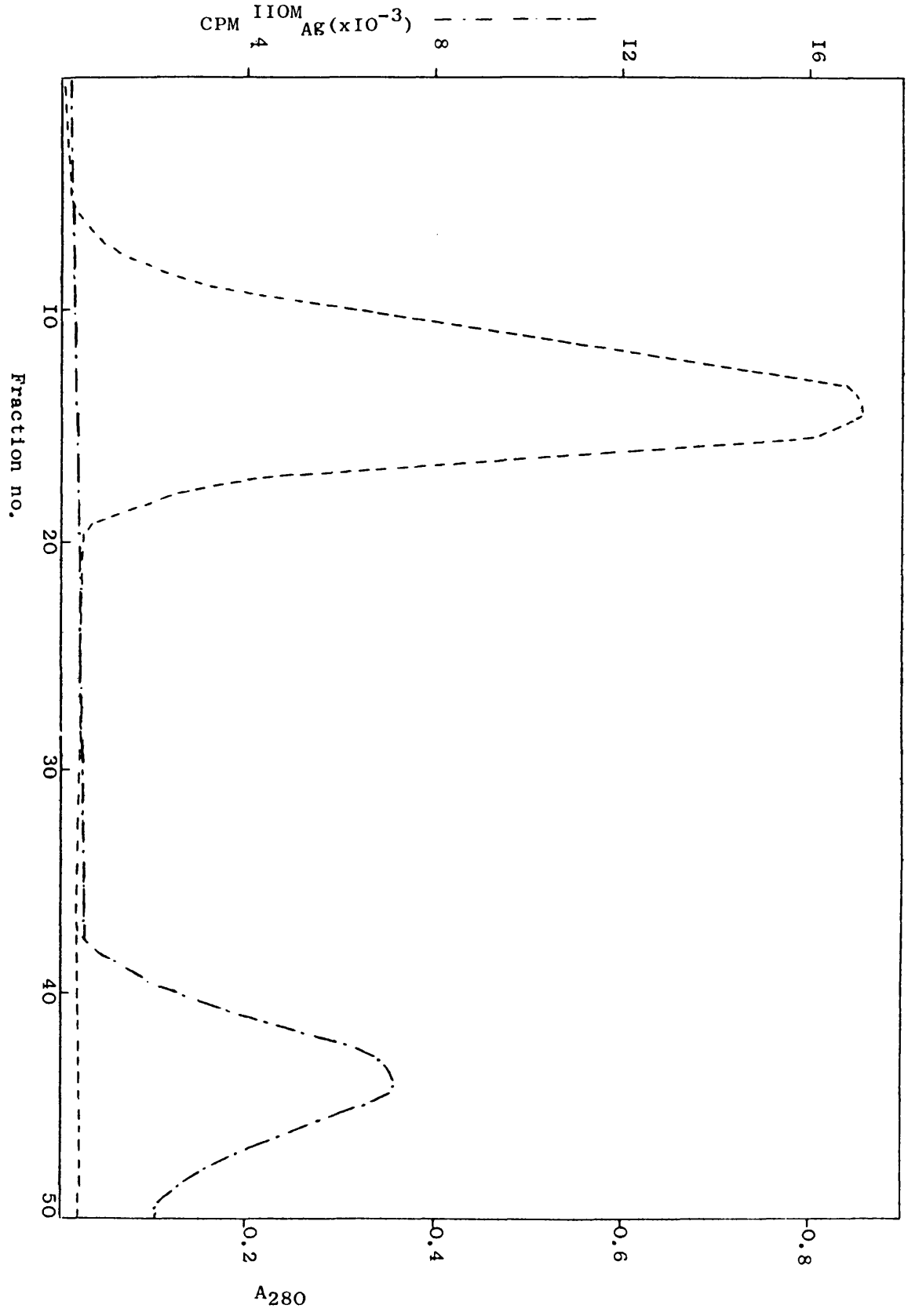

CPM IIOM Ag(xIO-3)
4
8
I2
I6
IO
20
30
40
50
Fraction no.
0.2
0.4
0.6
0.8
A280

Figure 8.2.

Biogel P4 column chromatography of oxidised reductively aminated purified histone from wheat embryo.

Wheat embryo histone was isolated, oxidised, reductively aminated and finally metallated with $^{110M}AgNO_3$ (Section 8.2, page 132-133). The treated histone was then applied to a Biogel P4 column (50cm x 0.9cm) which was eluted with 1M sodium acetate pH 6.5. The radioactive content of each fraction was monitored in an LKB-Walloch gamma counter.------

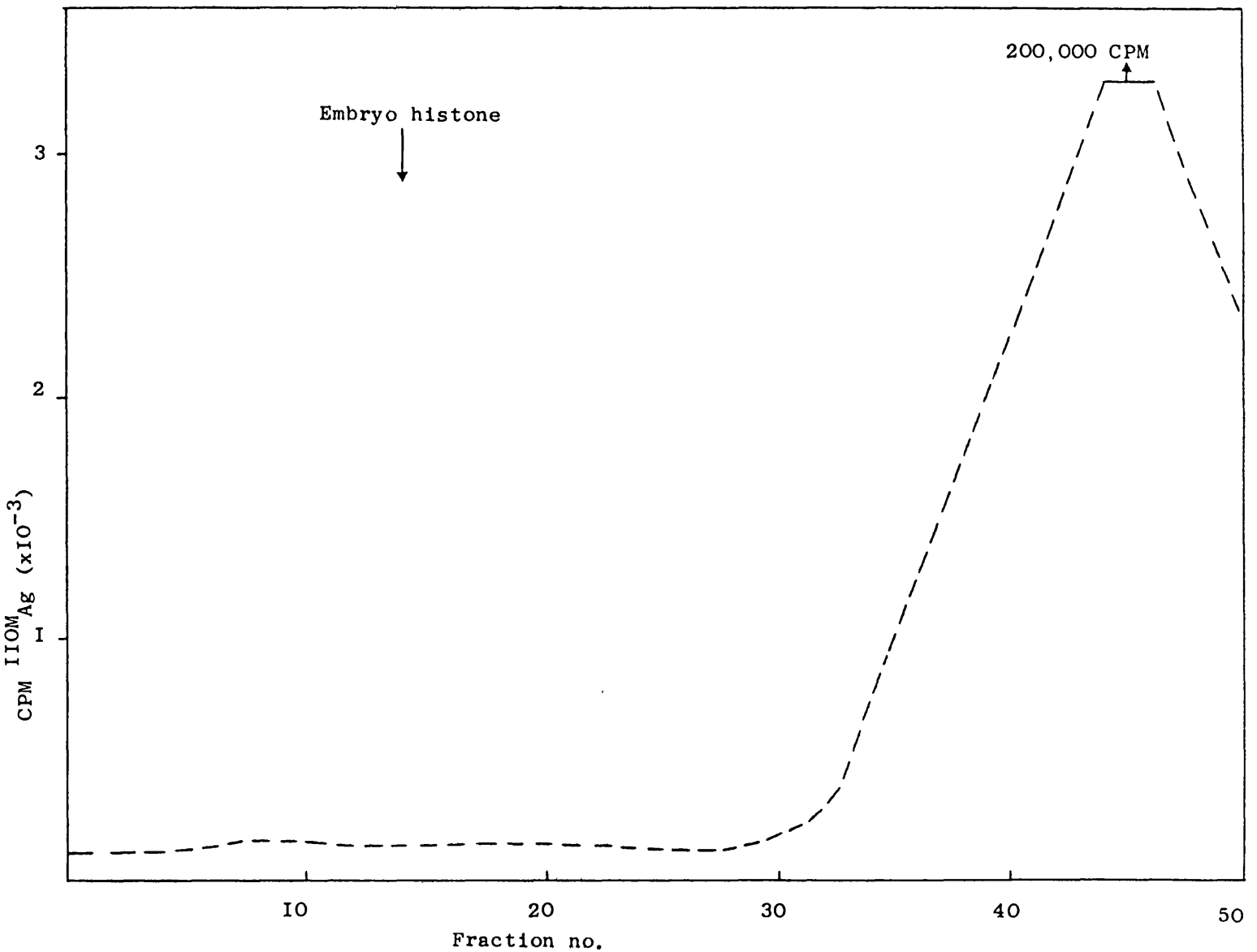
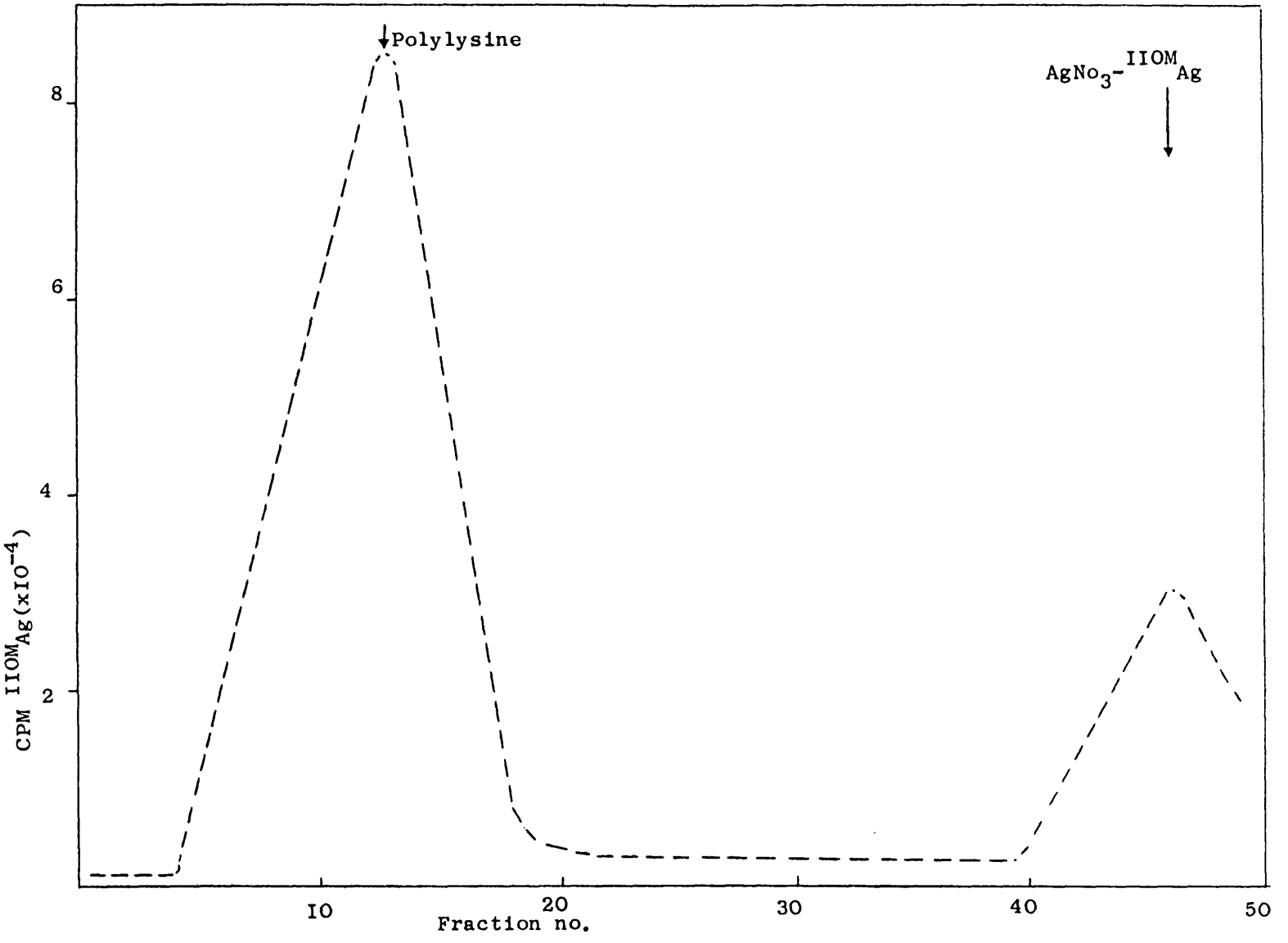

200,000 CPM
Embryo histone
3
2
1
CPM IIOMAg (xIO-3)
IO
20
30
40
50
Fraction no.

Figure 8.3.

Biogel P60 chromatography of ADP-ribosylated polylysine oxidised and labelled with silver 110M

ADP-ribosylated polylysine (page 135) was oxidised and reductively aminated with 4-amino thiophenol. The product was reacted with an excess of $^{110M}AgNO_3$ (page 132). The mixture was applied to a Biogel P60 column 50cm long and 0.9cm diameter. The column was eluted with 1.0M sodium acetate pH 6.5. Radiolabel was determined by counting 200µl aliquots of each fraction in an LKB-Walloch gamma counter.


Polylysine
AgNo3-IIOMAg
8
6
4
2
CPM IIOMAg(xIO-4)
10
20
30
40
50
Fraction no.

Conclusion

The early enzymological studies showed that *in vitro* work using isolated nuclei is not meaningful. The underlying problem was that, though pure nuclei could be isolated, the synthesis and breakdown of the polymer/monomer was difficult to interpret. The ADP-ribose transferase and glycohydrolase are difficult to study because of the unknown amount of endogenous ADPR/polymer in the nuclei. The chemical (base) catalysed hydrolysis of the ADPR/protein link contributed to this difficulty. No doubt conditions could have been found which separated the desirable (enzyme) and undesirable (base and other enzymes) reactions. Such studies would have involved at least partial purification of the enzymes and a reconstituted system. The chemical hydrolysis of the protein-polymer link would still have to be solved.

An interesting finding was that the synthetic and degradative activities could be separated by repeated freeze-thawing. However this was complicated by the fact that although the glycohydrolase was destroyed by freezing, the total amount of incorporation of [^{3}H]NAD into acid insoluble ADP-ribose did not rise, indeed it fell. This was unexpected and the tentative conclusion is that freeze-thawing destroys the nucleus, causing aggregation of the DNA and protein, which then masks some of the ADP-ribose transferase, or the acceptors, or both.

It seems obvious therefore that the enzymic studies

in vitro, would not contribute to the overall brief of this work - that is, to determine the biological role, if any, of protein ADP-ribosylation during seed germination. It was at this time that various ADP-ribose transferase inhibitors were being developed in this laboratory. A study was made to see if any of these had a detectable effect on either the rate of germination or the viability of embryos. None of the ADP-ribose transferase inhibitors has any discernable effect on either of the above parameters. The use of radiolabelled nicotinamide and thymidine showed that the inhibitors penetrated the embryos and entered the cells. Thus the lack of a physiological effect could not simply be due to the impermeability of the embryo to these compounds. It must be assumed that either the ADP-ribose transferase may be inhibited without effect on the embryo or there is a sufficiently small percentage (perhaps 0.1% or less) of active enzyme which maintains a very small but vital amount of ADP-ribosylated proteins. This possibility can only be investigated by directly observing the effect of such inhibitors on the level of ADP-ribosylated proteins in vivo. Such a study is not feasible until a satisfactory analytical method for determining ADP-ribosylated proteins is developed.

Part of the problem with these inhibitor studies is the lack of published information about plant biochemistry. e.g. hydroxyurea had no effect on the rate of germination

of the embryos. In animal systems, hydroxyurea inhibits DNA replication (Yarbro, 1967). The evidence from this work is that the DNA replication in wheat embryos is unaffected by hydroxyurea. Finally, in the inhibitor studies, cordycepin was used in an attempt to inhibit DNA replication so that DNA repair could be studied. As expected, the cordycepin was a powerful inhibitor of DNA replication. However as a specific inhibitor there were definite problems with cordycepin. Firstly RNA synthesis will also be inhibited. This may not have proved to be a problem because cellular effects produced by the inhibition of RNA synthesis can be allowed for. A serious drawback with cordycepin in its possible incorporation into the NAD pool. The 3-deoxy grouping in cordycepin is unlikely to inhibit NAD synthesis. In fact there is no obvious reason why 3-deoxy adenosine triphosphate (cordycepin triphosphate) may not be a substrate for NAD pyrophosphorylase since 2-deoxy adenosine triphosphate is a good substrate for this enzyme (Morton, 1958).

The inter ADP-ribose links in poly(ADP-ribose) are ribose-ribose 1'-2', so it can be seen that the cordycepin analogue of NAD is very likely to be a substrate for ADP-ribose transferase, with unpredictable results. Furthermore one cannot predict whether or not such a 3-deoxy adenosine containing polymer will be a substrate for poly(ADP-ribose) glycohydrolase.

There can be no doubt that mono ADP-ribosylation of protein in embryos occurs because it is seen on caesium chloride urea gradients. However it is obvious that this material is not ADP-riboslyated histone. The problems of analysing such modified non-histone proteins are enormous. Perhaps the main problem is that the non-histone proteins are simply all those nuclear proteins which are not histones. Therefore one must expect some thousands of different proteins in this class.

The final conclusion to this work must be that there is no obvious relationship between ADP-ribosylation of wheat embryo protein and germination. A successful solution of this problem will have to await development of suitable analytical systems for protein ADP-ribosylation in vivo.

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CHAPTER 9

Appendix

DNA Repair in Wheat Embryos

Preliminary Studies

9.1. Introduction

Knowledge of DNA repair in higher plants is very poor when compared with that known for bacteria or mammalian cells. Previously, excision repair was believed to be absent in plants until Veleminsky & Gichner, (1978) reported the occurrence of photoreactivation of UV-induced pyrimidine dimers in cells of Nicotiana tabacum and carrot, the excision of dimers in protoplasts of carrot and in embryos of Lathyrus sativus, and the repair of single-strand DNA breaks induced in carrot protoplasts and barley embryonic cells by ionizing radiation. In irradiated barley embryos unscheduled DNA synthesis and a greater accessibility of induced primers to E.coli DNA polymerase 1 were observed to occur preferentially in G_1 cells containing diffused chromatin. These reactions were inhibited by caffeine and EDTA. Unscheduled DNA synthesis was also observed in synchronised irradiated root cuttings of vicia faba and in barley embryos treated with 4-nitroquinoline oxide, the latter reaction being inhibited by caffeine and hydroxyurea. The occurrence of repair synthesis was also established in barley embryos treated with the mutagen, N-methyl-N-nitrosourea, under conditions which postponed the onset of germination after

such treatment. The same conditions enhanced the repair of DNA single-strand breaks induced by this mutagen and several other monofunctional alkylating compounds.

The problem with DNA repair studies in plants is that the cell walls are rather effective in preventing entry of compounds into the cells, be they macromolecular precursors or alkylating agents. This is possibly the reason why the study of plant cell DNA repair has fallen so far behind study of DNA repair in other eukaryotes and prokaryotes. It was thought that one might overcome at least part of this problem by using a physical method to damage the DNA. Various experiments can then be designed to follow the repair of such damage.

A study was therefore initiated into the effects of sonication on germination in the wheat embryo.

No previous work on DNA damage and repair has been carried out on wheat embryos, although much work has been done on barley seeds and embryos, and it would not be unreasonable to expect similar results in wheat embryos.

Work has been done on the effect of sonication on DNA but only *in vitro* and not *in vivo*, Richardson (1966) sonicated purified T_7 bactriophage DNA and showed, by the use of polynucleotide kinase end-group labelling with ^{32}P phosphate, that the single-stranded breaks produced had no base specificity; there was a reduction in molecular weight and the breaks were almost exclusively produced by

phosphodiester bond breakage. Previously, Richards and Boyer (1965) showed that this phosphodiester breakage gave rise to 5'-phosphoryl-terminated polynucleotides preferentially over 5'-hydroxy-terminated polynucleotides in a ratio of 16 to 1. Thus it seems that sonication produces single strand breaks repair of which might involve protein ADP-ribosylation.

It must be stressed that the work outlined in this chapter is qualitative and no attempt was made to make it quantitative. Indeed, quantitative data for DNA repair in any eukaryote has not yet been forthcoming because of the complexity of a system which is very poorly understood.

9.2. Method for Sonication Embryos

Embryos were sonicated in 5ml of germinating medium (1% w/v D-glucose; 0.01% w/v streptomycin sulphate) in a plastic test-tube (89mm x 17mm) packed in ice. An ultrasonics rapidas sonicator with a 3mm diameter probe was used at 20 KHz and 25 watts. Control embryos were soaked in the germinating medium (5ml) for one hour and sonicated embryos were soaked and then sonicated (in the same tube) for a total treatment time of one hour. After treatment the embryos were blotted dry before plating out for either germination or ^{3}H-thymidine labelling.

9.3 Velocity Sedimentation Analysis

The change in size of ^{3}H-thymidine labelled single-stranded DNA fragments was determined by velocity sedimentation through an alkaline sucrose density gradient by ultracentrifugation. Samples of twenty embryos were labelled for one hour (20µl ^{3}H-thymidine/vial) after various treatments and then homogenised as previously described (Section 3.2.2.)in one millilitre of 0.5M NaOH. The homogenate was spun in a MSE bench centrifuge for 15 minutes at 3,000 rpm, to remove the larger pieces of debris minimizing the possibility of bulk sample transfer through the gradient, and the supernatant was stored overnight at 4°C. 100 microlitres of the supernatant was carefully layered on top of a preformed 5 to 20% (w/w) linear sucrose gradient (0.1M NaOH) using a Gilson adjustable pipette, then centrifuged in a Beckman $SW_{50.1}$ at 49,000 rpm at 20°C (for the appropriate times) in a Beckman L-5 ultracentrifuge. The total gradient volume was five millilitres and was generated stepwise from 5, 10, 15 and 20% sucrose solutions (all in 0.1M NaOH) in cellulose nitrate centrifuge tubes and left overnight to diffuse into a linear gradient before use.

Gradients were fractionated from the bottom of the tube to the top by means of a stainless steel tube, held down the centre of the tube to the bottom by a perspex

holder which also held the tube; the gradient was pumped out of the tube by using an LKB multi-speed peristaltic pump and fractionated as follows: three drop fractions were collected onto TCA impregnated filter paper discs, which were then dried in an oven at 65°C for one hour, washed in batches four times in ice-cold 5% TCA (400 millilitre/100 discs) for 30 minutes each time and then dried in an oven at 65°C for one hour and counted (see Section 3.2.4.).

Section 9.4. Results

9.4.1. Embryo Viability

Figures 9.1 and 9.2 show the results of the effect of various sonication times on the viability of 50 embryos (100 embryos for the control samples) from two independent experiments. The embryos that had been sonicated for 30 minutes or longer were very soft and fragile.

9.4.2. [^{3}H] Thymidine Gross Incorporation

Figure 9.3 shows the gross incorporation of [^{3}H] thymidine into samples of five embryos, sonicated for various times, over a period of five hours and it is expressed as TCA insoluble radioactivity/embryo/five hours.

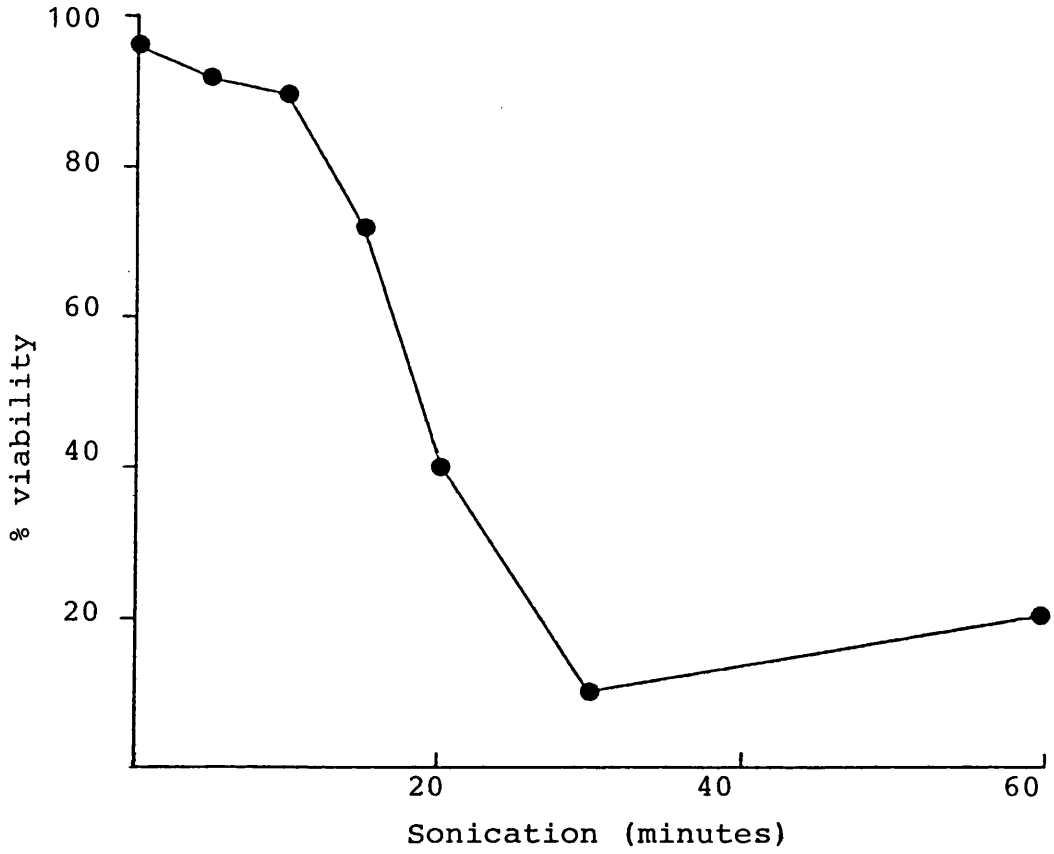

Figure (9.1) The effect of sonication treatment on the germination viability of wheat embryos.

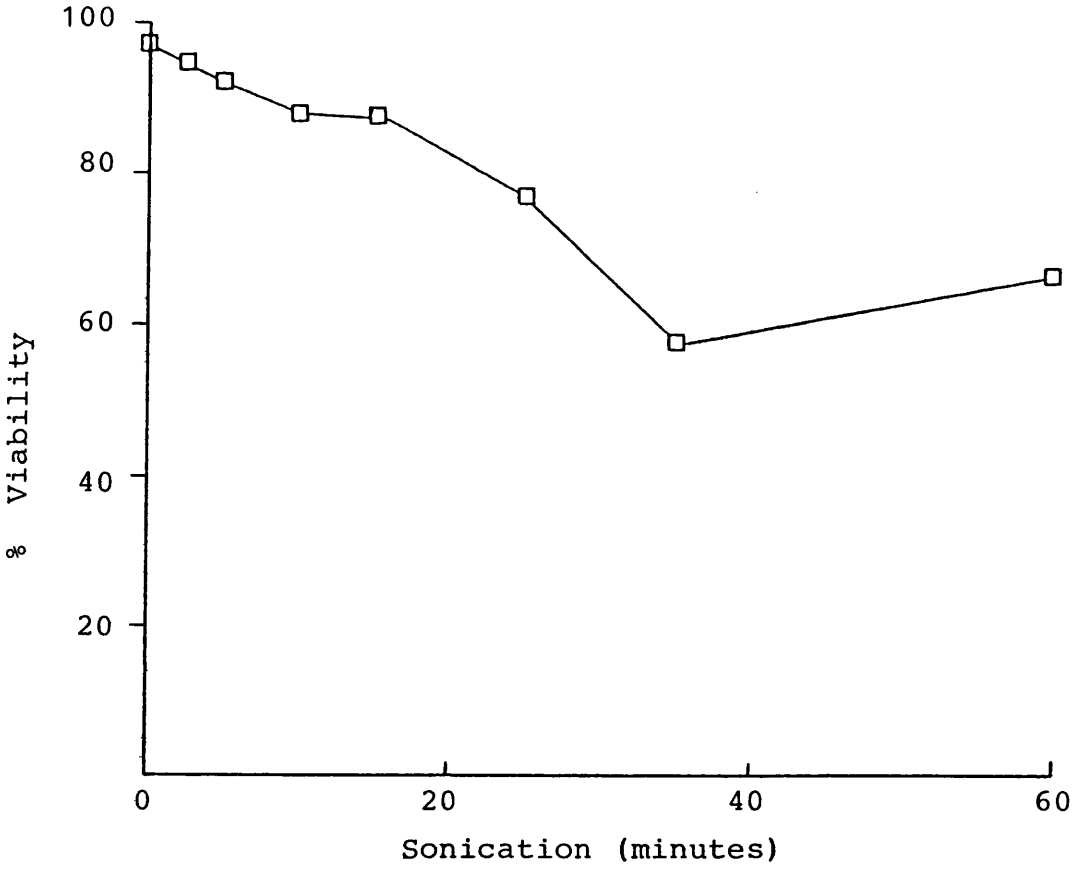

Figure (9.2) Effect of various sonication treatment times on the germination viability of wheat embryos.

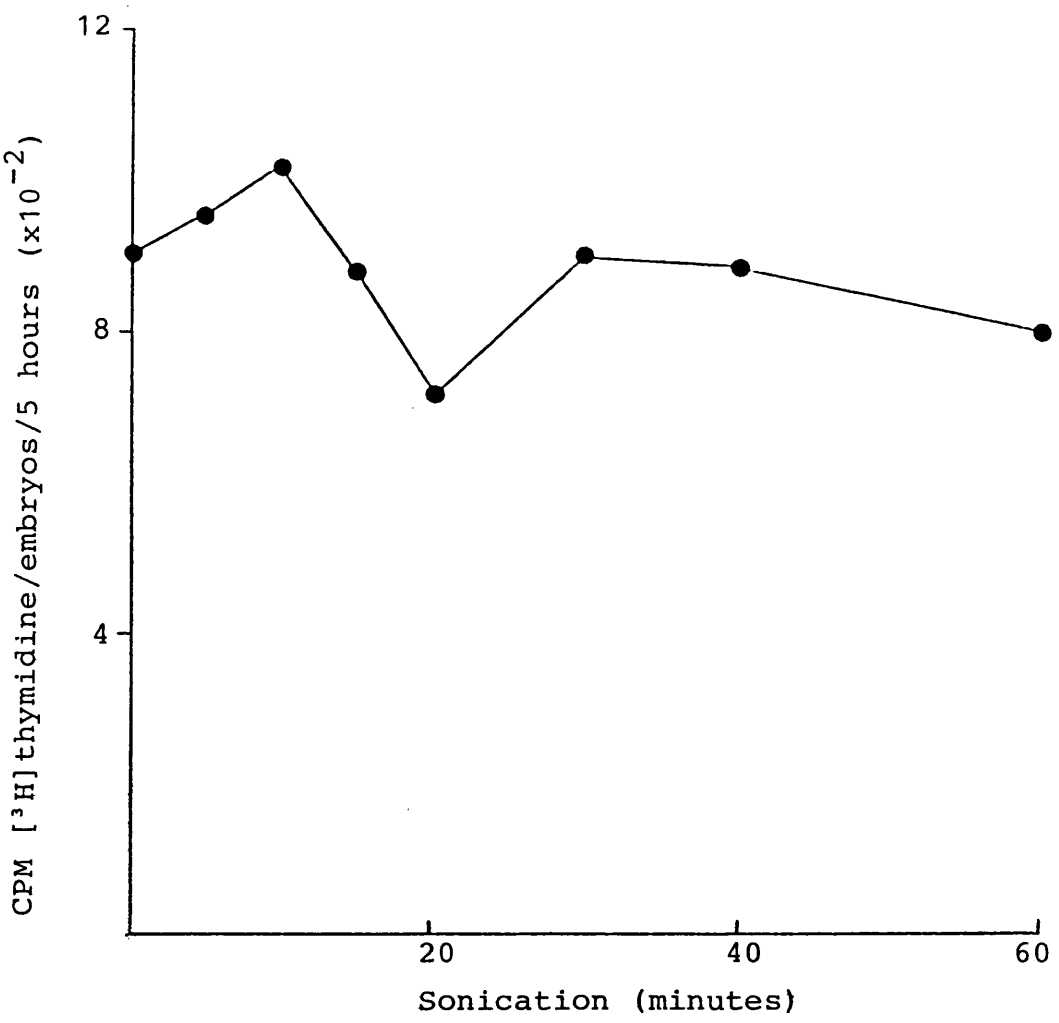

Figure (9.3) The gross incorporation of ^{3}H-thymidine into wheat embryos.

9.4.3. [^{3}H] Thymidine Incorporation Rate

Table 9.1 shows the incorporation of [^{3}H]thymindine into embryos, immediately after and four hours after treatment as TCA insoluble radioactivity/embryo/hour. Figure 9.4 shows the results of sonication treatment on embryo [^{3}H]thymindine incorporation rate which is calculated as the incorporation at four hours minus that at zero hours.

9.4.4. Velocity Sedimentation Analysis

Figure 9.5 shows the results of velocity sedimentation analysis on two samples run simultaneously, an unsoicated control and a sonicated sample (20 minutes sonication), the gradients were fractionated as discussed in Section 9.3. The results in Figure 9.5 are four drop fractions and a centrifugation time of 285 minutes.

The results in Figures 9.6, 9.7, 9.8 and 9.9 were obtained using the same method (see Section 9.3) of fractionation and a centrifugation time of 180 minutes, the samples received post-sonication incubation times of 0, 1, 3 and 6 hours respectively, before [^{3}H] thymidine labelling and homogenisation. For each figure, there was an unsonicated control sample and a sonicated sample (ten minutes sonication), aliquots (20 microlitre) of the homogenates were also treated and counted to determine

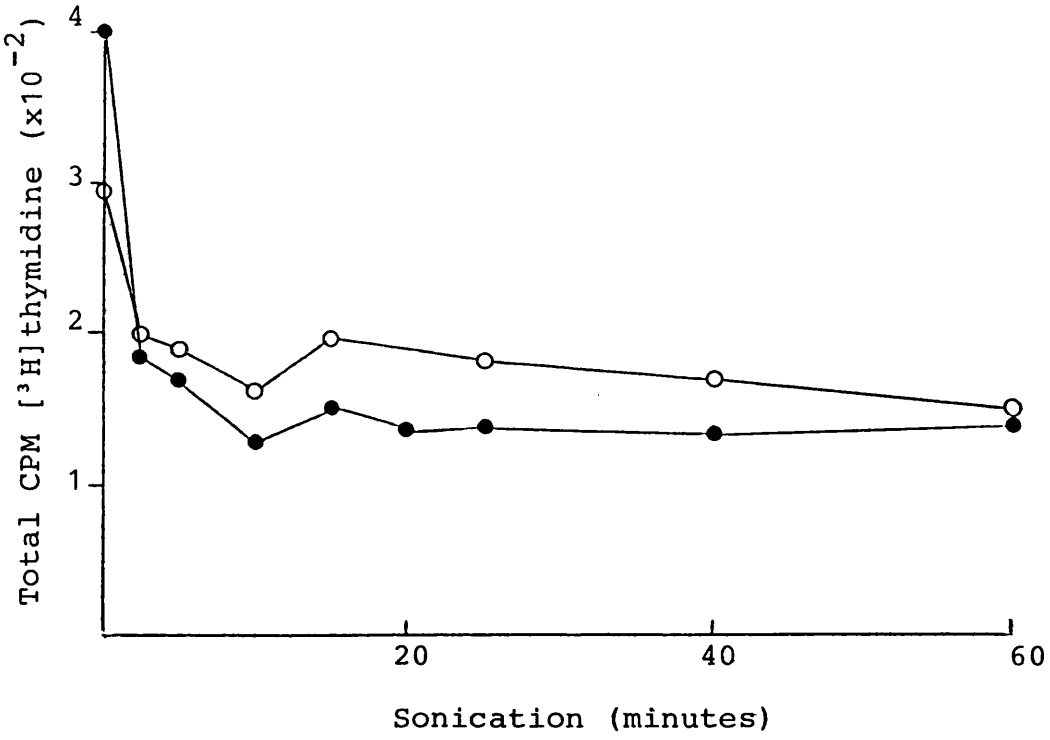

Figure (9.4) The incorporation rate of [³H]thymidine of sonicated embryos.

●——● 0 hours incubation

○——○ 4 hours incubation

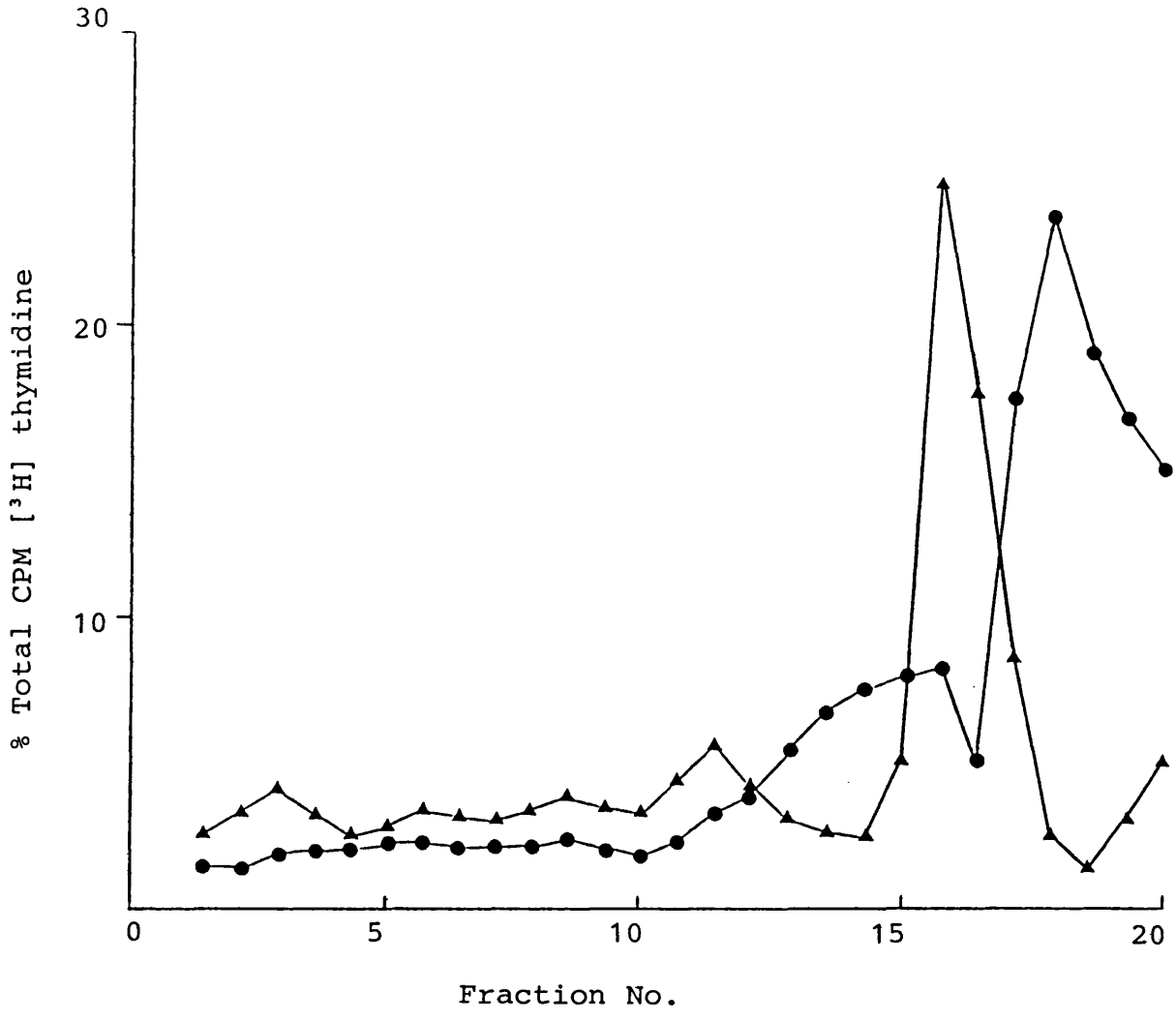

Figure (9.5) The velocity sedimentation analysis of alkaline sucrose density gradient ultra-centrifugation of sonicated and unsonicated wheat embryos.

●——● control

▲——▲ sonicated

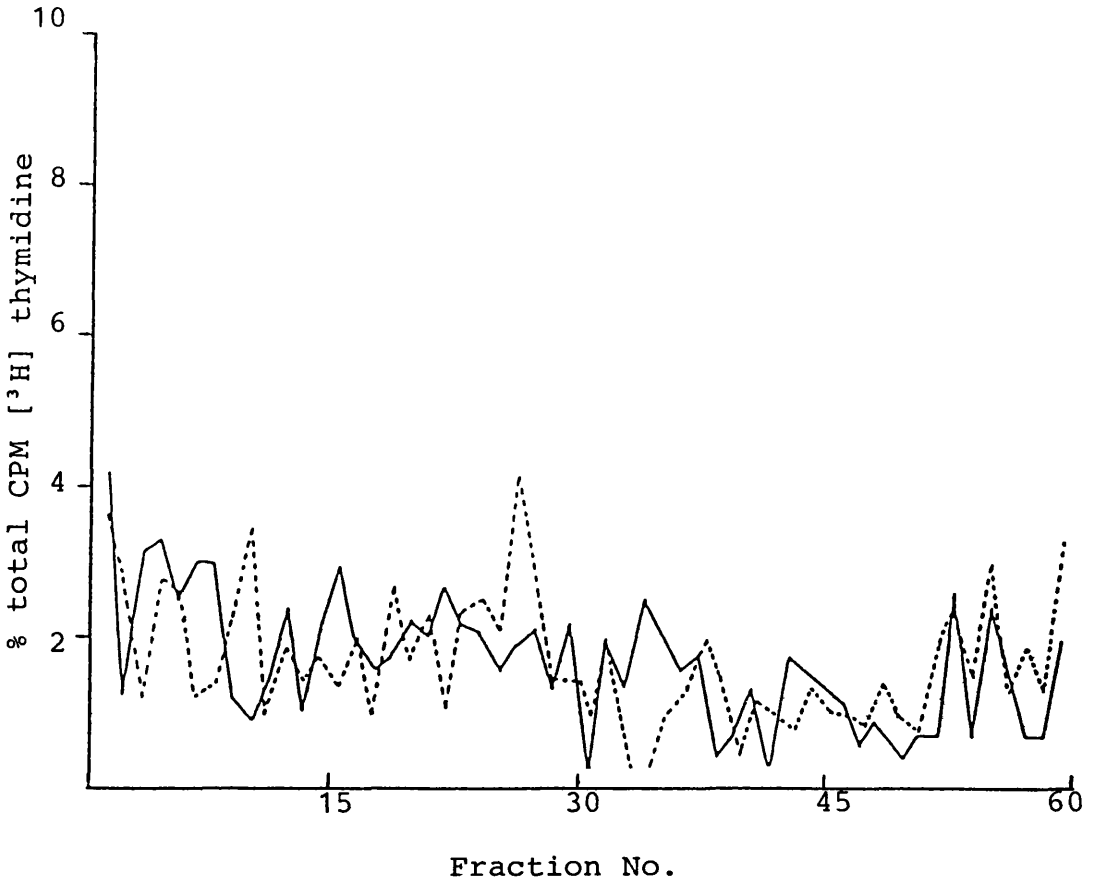

Figure (9.6) The 0 hours post-sonication incubation

——————— control

- - - - - - - - somicated

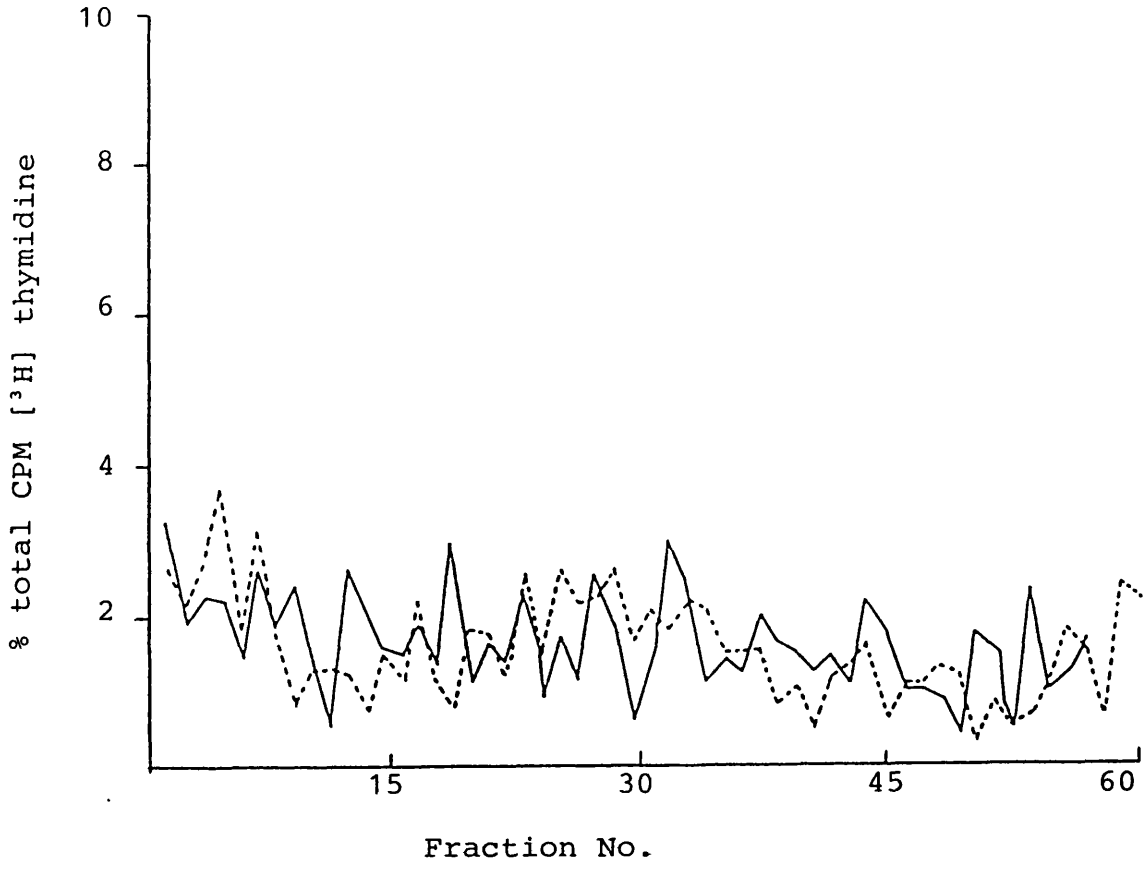

Figure (9.7) The 1 hour post-sonication incubation

——————— control

-------- sonicated

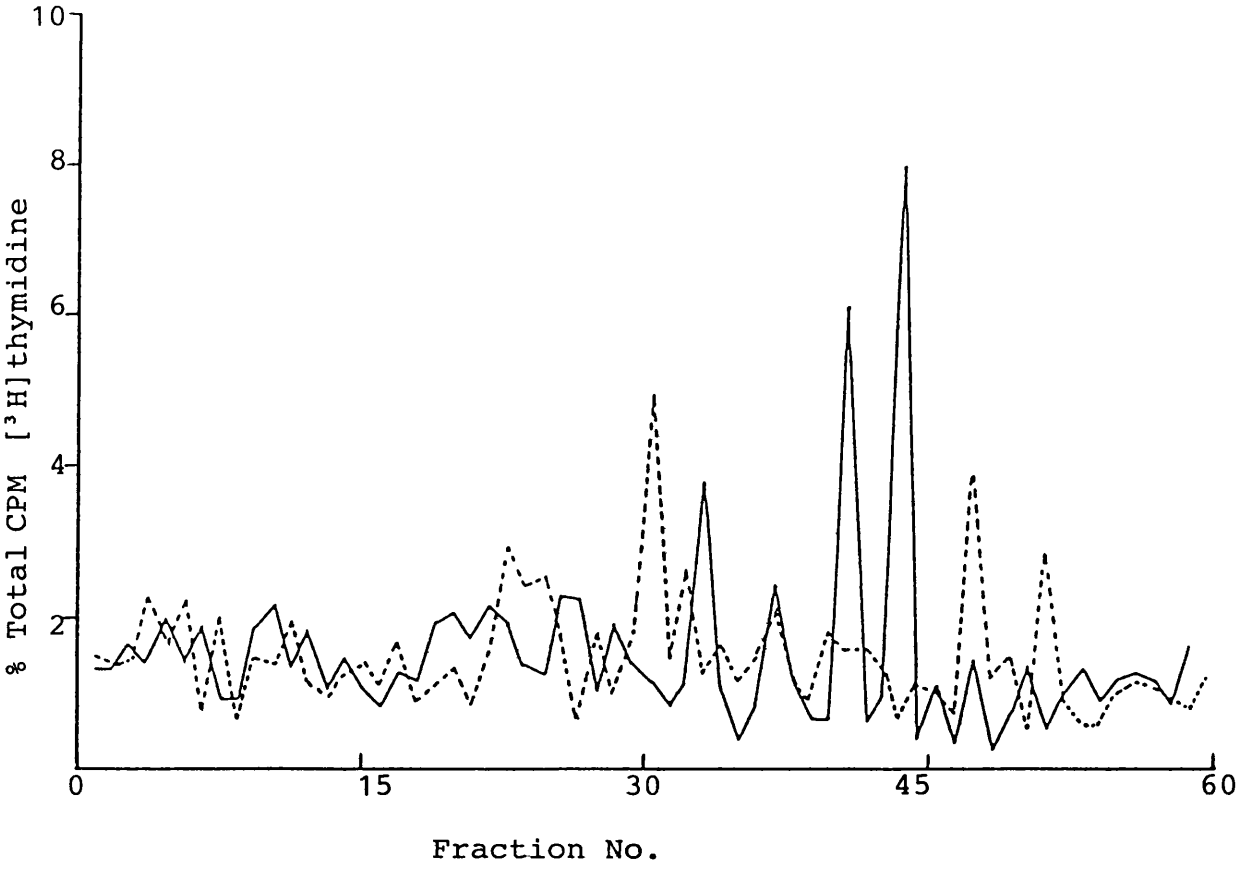

Figure (9.8) The 3 hour post-sonication incubation

——————— control

- - - - - - - - sonicated

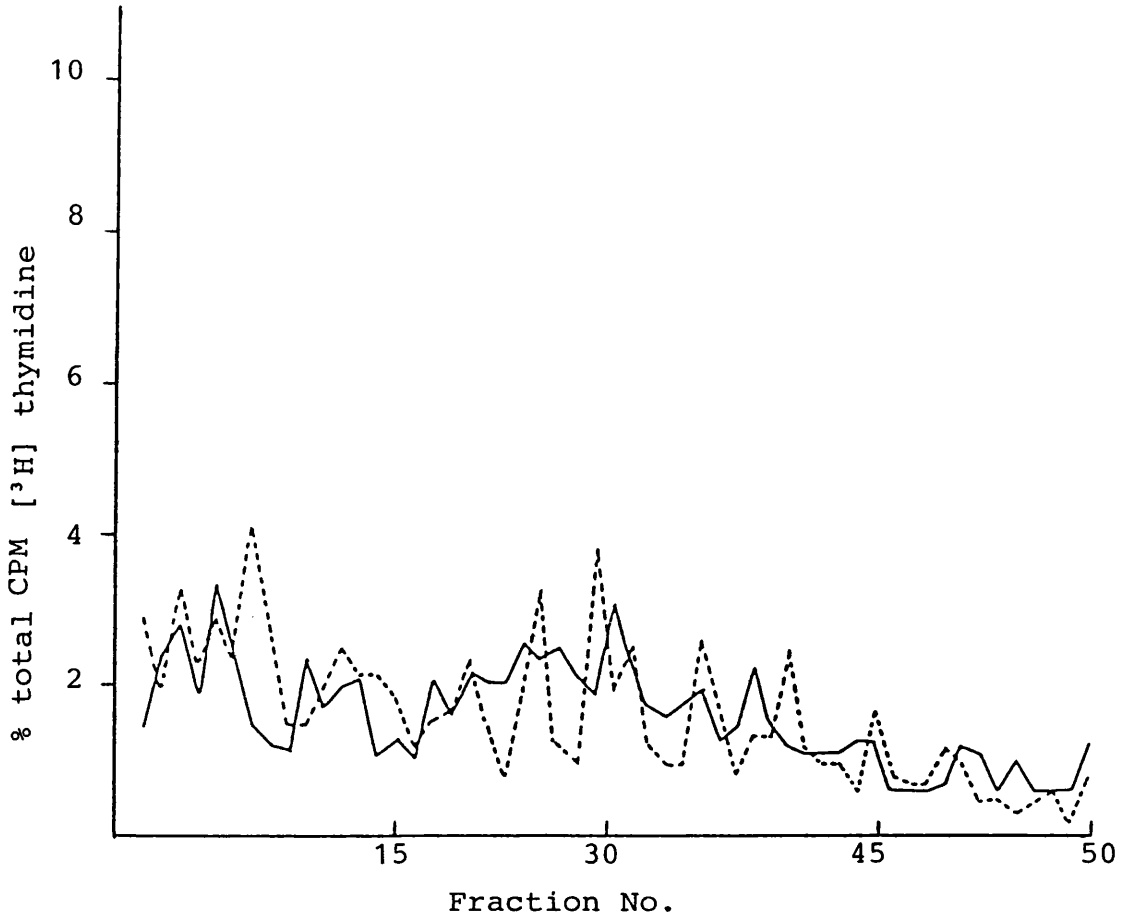

Figure (9.9) The 6 hour post-sonication incubation

———— control

- - - - - - - - somicated

total [^{3}H]thymidine incorporation and these results are plotted in figure 9.10.

In all experiments the relevant background counts were subtracted from the experimental values and the results were expressed as a percentage of the total radioactivity present in each gradient. The direction of sedimentation in all the figures was from left to right.

9.5. Discussion of the Results

The result in Figure 9.1 and 9.2 clearly show a loss of germination viability with sonication treatment. This is consistent with previous experimental observations and could be caused by mechanical damage to cellular components such as DNA or the protein synthesising ability of the embryo. The shape of the two graphs is similar with the values for treatment times of ten minutes or less being identical, the discrepancy between the values at longer treatment times is probably due to inherent variability in the effect of sonication on the gross structure of the embryo during prolonged treatment. The minimum on both graphs at 30 minutes treatment time is due to some indeterminate fractor(s). Damage to the embryos DNA is a possibility since Cheah and Osborne (1978) established that there is a relationship between the number of single stranded breaks in DNA and the loss of germination viability in rye seeds.

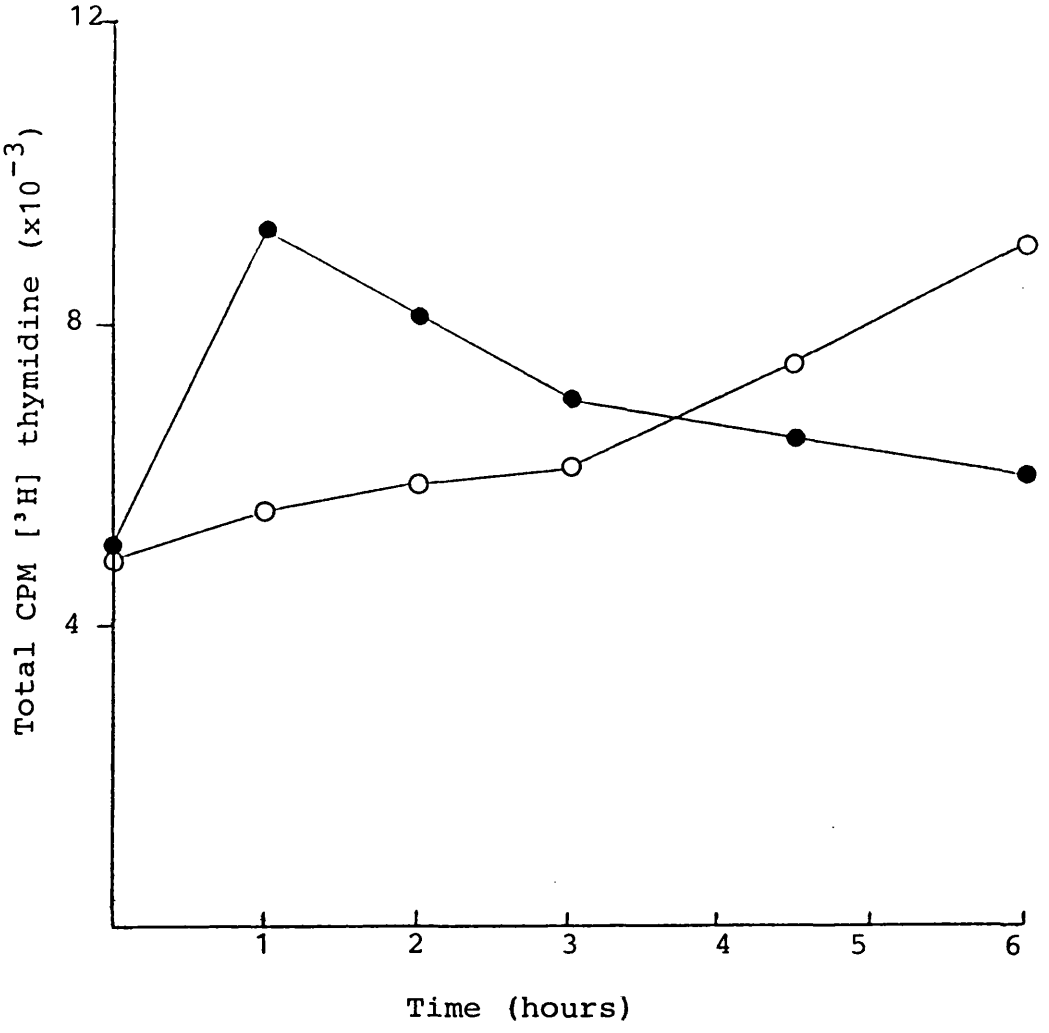

Figure (9.10) The incubation of 10 minutes sonicated and unsonicated sample with [³H]thymidine.

○——○ control

●——● sonicated

The results in Figure 9.4 show that the sonication treatment inhibits [^{3}H]thymidine incorporation. This implies damage to, or inhibition of, the DNA repair systems involved. These results could also be explained by the ant agonistic action of sonication treatment on DNA damage, and damage to, or inhibition of, the DNA repair systems. This might cause a maximal rate of [^{3}H]thymidine incorporation at 20 minutes treatment time.

The results in Figure 9.3 are not consistent with those in Figure 9.4 because the gross incorporation over five hours does not decrease significantly with sonication treatment. This might be due to a large statistical variation in the data since only five embryos were used for each time point. The rate of [^{3}H]thymidine incorporation in the control samples is negative. This means that the rate of unscheduled DNA synthesis was at a maximum sometime during the first hour of embryo soaking. Early repair of DNA damage after imbibition of water in rye embryos has been shown (Osborne et al., 1981) where the damage was caused by endonuclease action in the dry state of the embryo.

Alkaline sucrose density gradient velocity sedimentation analysis of the [^{3}H]labelled DNA was used to estimate the molecular size of single-stranded fragments of the DNA. Hence any change or difference between samples is due to a change in the number of single-stranded breaks

and/or alkali-labile sites such as those caused by base loss (Lindahl and Anderson, 1972). In the case of DNA damage induced by ionizing radation 20% to 50% of the detected breaks are believed to be due to alkali-labile sites (Paterson et al., 1973; Town et al., 1973). Thus, the results in figure 9.5 show that sonication treatment results in a relative increase in DNA damage (single-stranded breaks and/or alkali-labile sites) over the control samples. This does not preclude other types of damage but they are the most probable form since Richards and Boyer (1965) demonstrated that sonication of purified DNA gave rise to single-stranded breaks almost exclusively by phosphodiester bond rupture (not by carbon-carbon bond rupture).

The results in figures 9.6, 9.7, 9.8 and 9.9 were from experiments intended to investigate whether there is any repair of the induced single-stranded breaks within six hours of sonication treatment. If repair did occur one would expect a movement of the peaks in the sonicated samples towards the peaks in the control samples with increasing post-sonication incubation time. There would also probably be a concommitant increase in heterogeneity of the DNA which would be manifested by an increase in the width of the peaks in the sonicated samples towards that of control samples. However, the obtained data do not exhibit any significant peaks except in figure 9.8 where there are a number of small, spurious peaks in unexpected

positions (i.e. peaks in the sonicated sample occur above and below those in the control sample). Thus it is not possible to draw any conclusions as to the presence, or rate of, any repair of sonication-induced single-stranded breaks from this data.

The results in figure 9.10 are not consistent with those results in figure 9.4 as they show an increase in [^{3}H]thymidine incorporation over the six hours after soaking in the control samples whereas those in figure 4 show a decrease over the five hours after soaking. The reasons for this discrepancy are uncertain.

The conclusion from this study is that unscheduled DNA synthesis does occur in sonicated embryos but it is much depressed from control levels and so it appears that sonication not only damaged DNA but it also damages, or inhibits, some DNA repair systems. Unless an optimum treatment scheme can be found that eliminates, or at least minimizes this effect on repair systems sonication of wheat embryos is limited in its use as a tool for studying DNA repair in higher plants.

DNA Repair

Sonication time (Minutes)	incorporation rate [^{3}H] thymidine (CPM/hour/embryo)	
	0 hours	4 hours
0	398	297
1	166	199
5	120	162
10	143	197
20	108	177
30	112	161
60	96	102

TABLE 1

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